The Glycoprotein gp48 of Murine Cytomegalovirus

PROTEASOME-DEPENDENT CYTOSOLIC DISLOCATION AND DEGRADATION

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Degradation of misfolded or unassembled proteins that are co-translationally inserted into the endoplasmic reticulum involves the cytosolic proteasome system. Different principles may exist for the export of proteins into the cytosol for proteasomal degradation. Here we studied the degradation pathway of the viral glycoprotein gp48, a type I transmembrane protein, encoded by the m06 gene of murine cytomegalovirus. In cells stably transfected with the cytomegalovirus m06 gene or infected with the virus itself, two populations of gp48 can be distinguished that have different fates. Complexes of gp48 and the major histocompatibility complex (MHC) class I molecule, are transported to the lysosome for degradation. Unassembled gp48 is degraded by the cytosolic proteasome. Proteasomal inhibitors stabilize the unassembled gp48 in its core-glycosylated and membrane-associated form in the endoplasmic reticulum (ER)-Golgi intermediate compartment. This implicates that both endoplasmic reticulum and ER-Golgi intermediate compartment export gp48 and that degradation is coupled to a functional proteasome. Analysis of gp48 mutants revealed that the cytosolic part of gp48 was not responsible for the proteasome-dependent substrate transport out of the ER-Golgi intermediate compartment. Thus an indirect interaction between the proteasome and its substrate has to be discussed.

Secretary and membrane proteins are translocated into the endoplasmic reticulum (ER)† (1), where they are covalently modified, folded into their three-dimensional conformation (2) and, if necessary, assembled to oligomeric complexes prior to packaging into transport vesicles (3). A quality control machinery ensures that only functional molecules are deployed to their final destination (4–6). Misfolded or unassembled proteins are recognized (7) and eliminated from the ER by a proteolytic pathway coined ER-associated degradation (ERAD). For an increasing number of transmembrane and luminal proteins (8, 9–15) it has been demonstrated that the cytosolic ubiquitin-proteasome system is responsible for ERAD (16, 17). Thus membrane-spanning proteins, which do not pass the quality control machinery of the ER, must be transported back into the cytosol for degradation. Retrograde transport of proteins in yeast (10, 18, 19), and in mammalian cells (14, 15, 20) involve the Sec61 translocon. ER export of the MHC class I heavy chain through the translocon is accompanied by the removal of N-linked oligosaccharides (14, 15, 21).

The current model proposes that the export of MHC class I molecules out of the ER is independent of the catalytic activity of the proteasome. Accordingly, also in the presence of proteasomal inhibitors, deglycosylated degradation intermediates are accumulated in the cytosol. For US2- and US11-dependent degradation of the MHC class I heavy chain, the cytoplasmic tail of MHC class I heavy chain is required, although the dislocation does not require the proteasome (22).

Apart from the ER there may be other cellular compartments at which proteins are accumulated upon proteasomal inhibition. A mutated 46-kDa mannose 6-phosphate receptor is degraded by the proteasome even after leaving the Golgi compartment (23). Unassembled murine MHC class I molecules do not accumulate in the ER, but in vesicles assigned to the ER/cis Golgi (ERGIC) intermediate compartment and co-localize with components of the ubiquitin-proteasome system (24). More recently it was shown that mutated cystic fibrosis transmembrane regulator accumulates under these conditions near the centrosome in so called aggresomes (25) and co-localizes with components of the proteasome system (26).

This would predict that eucaryotic cells recognize proteins, which should not reach their final destination, in different compartments of the secretory pathway. Accordingly, multiple mechanisms may exist that ultimately lead to the breakdown of these proteins by the cytosolic proteasome.

The type I transmembrane glycoprotein gp48 encoded by the murine cytomegalovirus gene m06, interferes with the MHC class I pathway of antigen presentation (27). It binds to properly folded β2m-microglobulin (β2m-associated MHC class I molecules in the ER and transports them through the Golgi compartment into the lysosomes, where the gp48-MHC class I complex is degraded. To our knowledge the m06/gp48 gene is the most effective MHC class I regulating principle of MCMV.

To fulfill its function in vivo, gp48 has to be expressed in abundance to bind as many MHC class I molecules as possible. Consequently, situations should exist under which gp48 does not bind its physiological partner MHC class I. Here, we have studied the fate of such gp48 molecules. We demonstrate that gp48 molecules that fail to associate with MHC class I are degraded by a proteasome-dependent pathway. In vitro recon-
stition experiments showed that the cytoplasmic tail of gp48 is not required for the export of the protein out of the microsomal membrane, a process that is nevertheless proteasome-dependent.

** EXPERIMENTAL PROCEDURES **

**Cell Lines and Viruses—** NIH 3T3 cells (ATCC CRL1658) were transfected with the empty B45-Neo vector (28) or the constructs m06 and m06ACT, respectively (27), were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) newborn calf serum and 0.5 mg/ml G418 (Invitrogen). DLD-1 (ATCC CCL-221) human colon cancer cells were transfected with the empty B45-Neo vector or the constructs m06 were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% (v/v) fetal calf serum and 0.5 mg/ml G418 (Life Technologies, Inc.).

**Viruses and Plaque Assay—** BALB/c mouse embryonic fibroblasts were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml). These cells were used for virus reconstitution from recombinant BAC plasmids as described (29). NIH 3T3 cells (ATCC CRL1658) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) newborn calf serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml). These cells were used for propagation of recombinant viruses and immunoprecipitation. The MCMV wt strain used in these studies was the BAC-derived recombinant MW97.01 (30). Virus stocks were prepared and virus titers were measured by plaque assay performed on second-passage mouse embryonic fibroblasts monolayers as described (31).

**Generation of Recombinant MCMV Expressing a gp48-HA Fusion Protein—** For manipulation of the MCMV genome the MCMV genome has been cloned into an infectious bacterial artificial chromosome (BAC) in Escherichia coli (32). After transformation of the MCMV BAC plasmid pSM3fr, containing the complete MCMV genome, into permissive mouse cells MCMV was reconstituted, which has wild-type properties in vitro and in vivo (30). Homologous recombination of linear PCR fragments with the MCMV BAC plasmid pSM3fr in E. coli was applied to generate recombinant MCMV genomes (33). Construction of the MCMV genome with a COOH-terminal fusion of the HA-tag sequence (5'-TACCCCATACGAGTCCAGAATCACGGC-3') to the m06 gene: first a PCR fragment was generated by using the plasmid pACY717 (New England Biolabs) as template DNA and primers 5'-HA-m06 (5'-GTTAGAGCTAATCATACTACCTCCTCCCCGTTACAAATAACCCATACGCTTCCAGACGATCCTAGTACAGG-3') and m06-tag (5'-ACAGAGGACACGACCGAAGACGGAAGAC-3'). The resulting PCR fragment containing the Knf from pACY717, next to the HA sequence plus stop codon and finally on both ends homologies of about 50 bp up- and downstream to the native stop codon of the m06 gene within pSM3fr. This linear PCR fragment was then inserted into pSM3fr by homologous recombination in E. coli which simultaneously led to the selection of native m06 stop codon, to an in-frame fusion of the HA sequence plus stop codon to the m06 open reading frame and to insertion of Knf after the m06 gene. Correct mutagenesis of the resulting recombinant MCMV BAC plasmid pm06-HA-CAV was confirmed by restriction pattern analysis and sequencing (data not shown). After transfection of mouse embryonic fibroblasts cells with pm06-HA by the calcium phosphate precipitation technique (34) the recombinant MCMV m06-HA-MCMV, expressing the gp48-HA fusion, was generated.

**Antibodies and Reagents—** For detection of gp48 by immunoprecipitation the monoclonal antibody CROMA 229 (monoclonal antibody CROMA 229) (35) and the affinity purified rabbit serum m06-2 (5'-ACAGAGGACACGACCGAAGACGGAAGAC-3') and m06-tag (5'-ACAGAGGACACGACCGAAGACGGAAGAC-3'). The resulting PCR fragment containing the Knf from pACY717, next to the HA sequence plus stop codon and finally on both ends homologies of about 50 bp up- and downstream to the native stop codon of the m06 gene within pSM3fr. This linear PCR fragment was then inserted into pSM3fr by homologous recombination in E. coli which simultaneously led to the selection of native m06 stop codon, to an in-frame fusion of the HA sequence plus stop codon to the m06 open reading frame and to insertion of Knf after the m06 gene. Correct mutagenesis of the resulting recombinant MCMV BAC plasmid pm06-HA-CAV was confirmed by restriction pattern analysis and sequencing (data not shown). After transfection of mouse embryonic fibroblasts cells with pm06-HA by the calcium phosphate precipitation technique (34) the recombinant MCMV m06-HA-MCMV, expressing the gp48-HA fusion, was generated.

**Preparation of Microsomes—** For the isolation of microsomal membranes from ER and Golgi compartment 0.9 M sucrose was used. Cells were harvested with a cell scraper in a cell buffer (0.5 mM sucrose, 10 mM Tris, pH 7.4, 5 mM EDTA) and used at a final concentration of 100 μM. The protease inhibitor leupeptin was purchased from Amersham Biosciences (Braunschweig, Germany) and used at a final concentration of 4–6 μM. The protease inhibitors ALLN and MG 132 (Biomol) were added to cell culture at final concentrations of 100 mM and 40 μM, respectively. The final concentration of the lysosomotropic amine NH4Cl (Merck, Darmstadt, Germany) was 50 mM. The protease inhibitor leupeptin was purchased from Amersham Biosciences (Braunschweig, Germany) and used at a final concentration of 4–6 μM. Chloroquine (Sigma) was used at a final concentration of 100 μM.

**Metabolic Labeling and Immunoprecipitation—** Immunoprecipitation was performed as described previously (16). In brief, subconfluent layers of cells were pulse-labeled with [35S]methionine and [35S]-cysteine (Amersham Biosciences) and SDS-PAGE were carried out as indicated. After washing the membranes with PBS, cells were incubated in 0.2% gelatin in PBS for 45 min. After extensive washing in PBS, cells were incubated for 45 min with fluorescein- or rhodamine-conjugated goat anti-mouse, goat anti-rat, or goat anti-rib IgG, respectively (Dianova, Hamburg, Germany), in 0.2% gelatin. After repeated washing the coverslips were mounted on glass slides with Histofane (Cannon, Germany). The mounted cells were analyzed with a laser scanning confocal microscope (Leica, Heerbrugg, Switzerland; microscope DMIRE2; scanner TC250).

**Western Blot Analysis—** Samples were suspended in Laemmli sample buffer containing 5% β-mercaptoethanol and electrophoresed on 10% SDS-PAGE gels. Proteins were transferred onto Hybond-P membranes (Amersham Biosciences Inc.) in the presence of blotting buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3). Membranes were blocked in TBS-T (Tris-buffered saline, 0.1% Tween 20) containing 5% nonfat dry milk for 30 min at room temperature and then incubated in TBS-T containing primary antibody at the desired concentration. Membranes were washed three times in TBS-T. Then the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. The proteins were visualized with the ECL-Plus system (Amersham Biosciences).
Then a flow-up sucrose gradient was built in SW 41 centrifuge tubes (Beckman, Germany) reaching from 1.2 to 0.5 M sucrose. All sucrose solutions were prepared in 10 mM Tris, pH 7.4. Samples were centrifuged at 4 °C at 100,000 × g for 2 h. The enriched Golgi fraction banded at about 0.8 M sucrose and the enriched ER fraction at about 1.15 M sucrose. Fractions were isolated and stored at -70 °C. This protocol was adapted from Tabas and Kornfeld (36).

RESULTS

Two Different Fates of gp48 in Fibroblasts Stably Expressing MCMV m06—The viral glycoprotein gp48 encoded by the MCMV gene m06 binds to MHC class I molecules and re-routes the complex to the lysosome for degradation (27). The degradation of gp48/MHC class I complexes can be prevented by lysosomal inhibitors like the weak-base amine NH₄Cl or the serine/cysteine protease inhibitor leupeptin resulting in the accumulation of a complex consisting of Endo H-resistant MHC class I molecules and Endo H-resistant gp48 molecules (27, 37). In the presence of the specific proteasomal inhibitor lactacystin no stabilization of MHC class I molecules was detected, which is in line with the lysosomal degradation of the gp48-MHC class I complex. However, we observed that lactacystin could inhibit the degradation of a gp48 subfraction (37).

We, therefore, hypothesized that this is another fraction of gp48 molecules which is not assembled with the MHC class I heavy chain and retained in the ER and degraded in a proteasome-dependent manner. To test this hypothesis we expressed m06 by the human colon carcinoma cell line DLD-1 which lacks β₂m and therefore cannot express correctly assembled MHC class I complexes. Since gp48 only binds to β₂m-assembled MHC class I heavy chains (27), only free gp48 should be detected in these cells lines. The immunoprecipitation with CROMA 229 (Fig. 1A), a monoclonal mouse antibody directed against gp48, showed two major bands migrating at 46 and 48 kDa, respectively, which represent the 5- and 6-fold glycosylated forms of gp48 (see also Fig. 2C). After a 6-h chase period gp48 was found degraded and the endosomal/lysosomal inhibitors leupeptin and NH₄Cl could not prevent this process. In contrast, the proteasome specific inhibitors MG132 and lactacystin stabilized a major fraction of the gp48 molecules. The stabilized molecules still contained the Endo H-sensitive high-mannose oligosaccharide side chains since digestion with Endo H resulted in gp48 molecules migrating at 35 kDa and representing the deglycosylated form of the protein.

Fate of gp48 during Viral Infection—MCMV is a species-specific virus and the functions of specific cellular genes have shown species specificity. Therefore, to test the biological relevance of the transfection experiment we studied whether lactacystin could also accumulate a fraction of gp48 during MCMV infection and in mouse cells able to form mature MHC class I complexes.

Mouse NIH 3T3 cells were infected with MCMV wt MW 97.01 and m06 HA-virus mutant containing the m06 gene as a HA-tagged protein at a multiplicity of infection of 0.5. This virus enabled us to precipitate the m06 gene product with the HA antibody 3F10 (compare MW 97.01 with m06HA molecules, Fig. 1B), nevertheless, the virus has wild type features with regard to the m06 gene function (data not shown). 16 hours post-infection cells were labeled and chased for the indicated periods of time in the presence or absence of lactacystin and leupeptin. Directly after the pulse gp48 is present in an Endo H-sensitive form. As reported for the wild type gp48 (27), 4 h later gp48 is stabilized by leupeptin in its Endo H-resistant form (gp48r) and associated with MHC class I molecules (indicated by arrowheads). Lactacystin, however, stabilized gp48 in an Endo H-sensitive form (gp48s) that is only seen during inhibition of the proteasome and not during inhibition of lysosomal degradation or chase without inhibitors (indicated by asterisk).

In these lanes MHC class I heavy chain and β₂m light chain are detectable. However, these MHC molecules are in fact not stabilized by lactacystin since the same amount of MHC molecules (indicated by arrowheads) is detectable after chase in absence of lactacystin (probably due to pre-existing labeled MHC molecules in complex with gp48 molecules synthesized after the pulse) is not enriched by proteasomal inhibition.

Therefore, we concluded that in cells stably expressing m06 or in MCMV-infected cells, gp48 molecules have two different fates. gp48 bound to MHC class I molecules passes through the Golgi, acquires Endo H-resistance, and is transported to the lysosome. Free gp48, however, that is not assembled with MHC class I molecules is retained in its core-glycosylated, Endo H-sensitive form (gp48s). As reported for the wild type gp48 (27), 4 h later gp48 is stabilized by leupeptin in its Endo H-resistant form (gp48r) and associated with MHC class I molecules (indicated by arrowheads). Lactacystin, however, stabilized gp48 in an Endo H-sensitive form (gp48s) that is only seen during inhibition of the proteasome and not during inhibition of lysosomal degradation or chase without inhibitors (indicated by asterisk).
form in a pre-Golgi compartment and degraded by a nonlysosomal mechanism, probably by the proteasome (Fig. 1C).

Specific Proteasomal Inhibitors Prolong the Half-life of Unassembled gp48—To study the fate of free gp48, we used stably transfected mouse fibroblasts as an appropriate model. Immunological reagents were prepared to discriminate between the two different fates of gp48. CROMA 229, a monoclonal mouse antibody, binds the COOH-terminal part of gp48 (27). The polyclonal rabbit antiserum m06-2 was raised against a peptide within the luminal part of the protein (Fig. 2A). CROMA 229 and the antiserum discriminate between populations of gp48 with regard to the assembly state to the MHC class I molecules (Fig. 2B). CROMA 229 precipitates both the complex of full-length gp48 with a molecular mass of 48 kDa associated with the MHC class I heavy chain and /H9252 2m, as well as free gp48. The studies regarding the fate of the MHC class I-gp48 complex as detected by CROMA 229 have been published (27). In contrast to CROMA 229, the antiserum m06-2 precipitates

**Fig. 2.** Lactacystin stabilizes gp48. A, proportional representation of the MCMV m06 encoded glycoprotein gp48. Enclosed is the signal peptide (SP), the luminal domain with the consensus sites for N-linked glycosylation (branched symbols), the transmembrane region (TM), and the cytoplasmic tail (CT). The striped box marks the peptide sequence used for the preparation of the antiserum m06-2. The gray box marks the binding site of the monoclonal antibody CROMA 229. B, comparison of proteins precipitated by mouse monoclonal antibody CROMA 229 and antiserum m06-2. NIH 3T3 cells stably transfected with the MCMV m06 gene were pulse-labeled and lysed. gp48 was precipitated by either CROMA 229 or m06-2. H-2 L4 molecules were precipitated with monoclonal antibody 28-14-8s. The precipitates were analyzed by SDS-PAGE. hc, MHC class I heavy chains; s, Endo H-sensitive; d, Endo H-digested. C, left panel: NIH 3T3 cells expressing gp48 and control cells were pulse labeled and chased for the indicated periods of time in the presence or absence of the proteasomal inhibitor lactacystin. gp48 molecules were precipitated with m06-2 from aliquots of Nonidet P-40 lysates. Samples were separated by SDS-PAGE. Right panel, partial Endo H digestion. NIH 3T3 cells expressing gp48 were pulse labeled for 40 min and precipitated with m06-2 and digested with Endo H for 12 h or mock treated. Subsequently samples were analyzed by SDS-PAGE. D, band intensities are quantified by densitometry and plotted as percentages of the signal at 0 h.
only free gp48 as indicated by the complete lack of co-precipitating MHC class I heavy chain and β₂m in the m06-2 lanes. Therefore, the antiserum m06-2 enabled us to study selectively the fate of unassembled gp48 molecules.

The stability of unassembled gp48 was followed over time (Fig. 2C). Lactacystin-treatment of the m06 transfectants doubled the half-life of gp48. In the presence of active proteasome, free gp48 had a half-life of about 90 min, demonstrating that the free protein is slowly degraded by the proteasome (Fig. 2D). The extension of the half-life did not result in the maturation of the core-glycosylated form or in assembly with MHC class I molecules. The same results were observed after treatment with the proteasome inhibitor MG132 and the less specific calpain I-inhibitor ALLN (data not shown).

In addition to the three main bands representing different glycosylation forms of gp48 (see also Fig. 2C, right panel, arrowheads) two bands occurred after 2 h of chase in the presence of lactacystin (Fig. 2C). One band migrating at 35 kDa (asterisk) represents the deglycosylated intermediate of the full-length protein (asterisk), whereas the other band at 20 kDa (arrow) is a not precisely defined deglycosylated degradation intermediate of the protein as demonstrated by Endo H digestion and Western blot analysis (data not shown; see also Fig. 3B). These degradation intermediates may either reflect the incomplete inhibition of the cellular proteasome due to low lactacystin concentrations or represent the unspecific activities of other cellular proteases. At higher concentrations of lactacystin the degradation intermediates are missing (see Fig. 3A). However, one should be aware that higher concentrations could also affect other cellular proteases (38, 39). Altogether, free gp48 is stabilized in the core-glycosylated, Endo H-sensitive form upon inhibition of the proteasome.

Lactacystin Stabilizes gp48 in a Pre-Golgi Compartment in a Membrane-associated Form—Hughes and colleagues (21) showed that for ERD unassembled MHC class I molecules are first transported from the ER into the cytosol, deglycosylated, and subsequently degraded by the proteasome. Therefore, inhibition of the proteasome results in the accumulation of deglycosylated MHC class I heavy chain in the cytosol. Because gp48 is stabilized in its glycosylated form upon treatment with specific proteasomal inhibitors, we tested whether gp48 is transported out of the ER during inhibition of the proteasome. The fate of m06 pulse-chase labeled in the presence of lactacystin was followed over time. Homogenates of metabolically labeled cells were separated in a soluble and a membrane fraction prior to immunoprecipitation. After pulse labeling the majority of gp48 molecules were detected in the membrane fraction in an Endo H-sensitive form and remained membrane-associated during the entire chase period of 4 h (Fig. 3A).

To establish the topological orientation of membrane-associated gp48, homogenates of metabolically labeled cells were separated in a soluble and a membrane fraction and subsequently treated with the protease proteinase K in a protease protection assay (Fig. 3B). Samples were either treated with proteinase K alone or with proteinase K and the detergent Nonidet P-40 and subsequent immunoprecipitation was performed with the antiserum m06-2. We expected that the protease treatment would degrade the cytosolic tail of proteins, but leave protected transmembrane and luminae domains intact. Treatment with proteinase K resulted in the generation of a product that differed in electrophoretic mobility by the size of 3 kDa that precisely reflects the contribution of the cytosolic tail (asterisk). Solubilization of the microsomes by Nonidet P-40 resulted in complete degradation of gp48. After a 4 h chase period the remaining gp48 was still reduced of its cytoplasmic tail by proteinase K. Thus, in the presence of lactacystin the stabilized molecules remained inserted into the membrane and only the COOH-terminal part of the protein is accessible to the protease. In the absence of lactacystin, no proteinase K-resistant gp48 remained after 4 h of chase.

Lactacystin-stabilized gp48 Accumulates in the ER/ER-GIC—The degradation kinetics of gp48 is slow when compared with that of other proteins of the secretory pathway that are degraded by ERAD (14, 15, 18). We therefore wondered whether the protein was still located in the ER or whether it had proceeded within the export pathway. To test this, we studied the intracellular distribution of gp48 in the presence or absence of lactacystin and leupeptin. To visualize gp48 we had to use CROMA 229 as primary antibody since the antiserum m06-2 specific for unassembled gp48 suffered from unspecific background problems. Therefore, in the following experiments both the assembled and unassembled form of gp48 are detected and the differences seen are due to the accumulation of one or the other form. After 4 h of chase in the presence or absence of lactacystin, we could detect an accumulation of gp48 in a reticular and vesicular structure in nuclear proximity (data not shown). Co-staining with compartment specific cellular markers identified the cellular compartments in which the different inhibitors accumulated gp48. To further characterize this structure, double staining of gp48 and cellular marker proteins were made in the presence or absence of lactacystin and leupeptin, respectively. It could clearly be seen that the localization of gp48 in the presence of lactacystin (Fig. 4, A, C, and E) differs from the localization in the presence of leupeptin (Fig. 4, D, F).
Proteasomal Degradation of a Viral Protein

Proteasomal Degradation Does Not Depend on the Cytoplasmic Tail of gp48—So far our data demonstrate that the transport of gp48 into the cytosol depends on a functional proteasome. Recently, Mayer and colleagues (40) showed that the proteasome itself could participate in the extraction of Sec62 derivatives from the lipid bilayer. In this example, a direct interaction of the proteasome with the cytosolic part of the substrate regulated the retrograde transport out of the lipid bilayer. To test whether the cytoplasmic domain of gp48 is required for the export of the protein to the cytosol, a mutant of gp48 (gp48ΔCT) was constructed. In this mutant the complete cytoplasmic tail is deleted, so that there is no cytosolic target left for the proteasome (Fig. 5A). This deletion also excludes the COOH-terminal ubiquitination of gp48. Since it is known that ubiquitination in yeast normally takes place in the cytosol (18, 19) this mutant of gp48 should indicate whether ubiquitination of the substrate is necessary for proteasomal degradation.

The mutant still interacts with the MHC class I molecules (27). After immunoprecipitation with the antiserum m06-2 three bands at the molecular mass of 41–45 kDa (Fig. 5B) were detected, which represent different glycosylation forms, as revealed by Endo H digestion (data not shown). The reduction in molecular weight of the mutant lacking the cytosolic tail corresponds to the predicted loss of 3,000. In the absence of a proteasomal inhibitor the half-life of gp48ΔCT of 90 min was comparable with that of the full-length protein (see Fig. 3B). Upon inhibition with lactacystin the half-life of gp48ΔCT doubled, as it was the case for full-length gp48. In the presence of low lactacystin concentrations we could also detect the deglycosylated full-length protein at 30 kDa as well as a deglycosylated degradation intermediate now migrating at 17 kDa, as revealed by Endo H digestion (data not shown). Since the gp48ΔCT degradation intermediates differ in size from the wild type intermediates the 3 kDa of the cytoplasmic tail, this led us to conclude that the 20- and 17-kDa intermediates represent COOH-terminal fragments (see Fig. 2C). The degradation intermediates as well as the fully glycosylated protein remained membrane-associated upon stabilization by lactacystin (data not shown). Taken together, the fate of gp48ΔCT is indistinguishable from the full-length protein with regard to the influence of the proteasomal inhibitors on half-life and topology of the rescued protein. This implicates that gp48ΔCT is also degraded in a proteasome-dependent manner and that the cytoplasmic domain of gp48 is not essential for proteasome-dependent re-translocation and degradation. The removal of all potential ubiquitination sites did not influence the degradation process.

Membrane Export of gp48 Is Initiated by the Proteasome but Does Not Depend on the Cytosolic Part of the Substrate—To provide direct evidence for the involvement of the proteasome in the transport process of gp48 and gp48ΔCT we reconstituted the components of the degradation system in vitro. Microsomes were isolated from stably transfected cell lines and characterized in Western blot analysis. We could detect the ER-resident proteins calnexin, calreticulin, and BiP in this microsomal fraction, whereas the Golgi or plasma membrane markers were not present (data not shown). Thus, the isolated microsomes mainly represented the ER fraction of cells expressing gp48 or gp48ΔCT. The preparation did not contain proteasomal activ-

B, D, and F). As already shown by Reusch and co-workers (27), leupeptin accumulated gp48-MHC class I complexes in lysosomes as defined by the lysosomal-associated membrane protein Lamp-1 (Fig. 4F), but not in the ER (marker calnexin) or ERGIC (marker p58; Fig. 4, B and D). In contrast, in cells treated with lactacystin, gp48 does not accumulate in lysosomes (Fig. 4E) but rather in the ERGIC compartment (Fig. 4C) and the ER (Fig. 4A). This indicates that during proteasomal inhibition gp48 accumulates in the ER and is also sorted to the ERGIC compartment.

These results show that the fate of MHC-assembled and noncomplexed gp48 is clearly different. The COOH terminus of gp48 contains the information for lysosomal targeting (27) which defined the fate of the MHC class I-gp48 complex. Free gp48 cannot use this information and is retained in the ER/ERGIC for degradation.

Fig. 4. gp48 is stabilized in the ER and ER intermediate compartment. NIH 3T3 transfectants were analyzed by confocal laser scanning microscopy. The proteasomal inhibitor lactacystin or the lysosomal inhibitor leupeptin was added for 6 h prior to immunostaining. A + B, double staining of cells with the ER marker calnexin (green) and gp48 (red) with monoclonal antibody CROMA 229 in the presence of leupeptin showed no co-localization and weak co-localization in the presence of lactacystin. C + D, double staining with the ERGIC marker p58 (green) and gp48 (red) shows a co-staining in the lactacystin-treated cells, but not in cells treated with leupeptin. E + F, double staining with the lysosomal marker Lamp-1 (green) and gp48 (red) demonstrates a co-localization with gp48 stabilized by leupeptin but not by lactacystin.
gp48 and gp48ΔCT could be reconstituted in vitro. Since lactacystin as a pro-drug does not work efficiently in in vitro experiments, the proteasomal inhibitor MG 132 has to be used to prevent degradation of gp48 and demonstrate that the proteasomal preparation was not contaminated with other proteases. The ER resident protein calnexin is present in the microsomal fraction but should not be accessible to the proteasome unless the system was leaky. Indeed this control substrate was not affected by the addition of proteasome to the reconstituted system.

These data indicate that the proteasome initiates the export of the substrate proteins from the microsomes. To exclude that the export of gp48 and gp48ΔCT occurs already after addition of ATP, the microsomal membranes containing m06 were first incubated with ATP for 4 h and then subjected to proteinase K digestion to test membrane topology of gp48 (Fig. 6B). Proteinase K digestion should degrade exported protein under these conditions. However, unless microsomal membranes were dissolved by Nonidet P-40, the molecule just shifted by the 3-kDa cytosolic tail when digested with proteinase K, indicating that the molecule remains inserted in microsomal membranes. These results demonstrate that the isolated microsomes, ATP, and proteasome provide all compounds that are necessary for proteasomal degradation of gp48 in vitro. In this in vitro system gp48 and gp48ΔCT are degraded by a functional proteasome, which is also necessary to initiate the transport of the substrate out of the membrane.

**DISCUSSION**

In pioneering studies on viral protein-induced MHC class I heavy chain degradation it was shown that US2 and US11, two glycoproteins encoded by HCMV, are responsible for the rapid degradation of MHC class I heavy chains by the proteasome (14, 15). Binding of the viral proteins to the MHC class I heavy chain leads to the transport of the substrate through the Sec61 translocon to the cytosol for proteasomal degradation. Further investigations clearly demonstrated that the proteasomal deg-

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**FIG. 5.** Mutant gp48 ΔCT degradation kinetics. A, schematic presentation of the mutant gp48ΔCT. The gray bar marks the transmembrane domain of gp48; the arrow indicates the terminal amino acid of gp48ΔCT. B, NIH 3T3 cells stably expressing gp48ΔCT and control cells (B45) were pulse-labeled in the presence or absence of lactacystin and chased for the indicated periods of time. gp48ΔCT was precipitated with the antisera m06-2 and analyzed by SDS-PAGE. C, band intensities were quantified by densitometry and plotted as percentages of the signal at 0 h.

**FIG. 6.** In vitro degradation of gp48 and gp48ΔCT. A, microsomes from NIH 3T3 cells stably expressing either gp48 or gp48ΔCT were prepared. Microsomal preparations were supplemented with 50 μM ATP, 20 μM MG 132, and purified 20S proteasome. The samples were incubated at 37°C for the indicated periods of time and then separated by 10% SDS-PAGE. Proteins were visualized with the indicated antibodies. B, for the proteinase K treatment of microsomes, samples were incubated in the presence of 50 μM triethanolamine-buffered ATP for the indicated periods of time. The samples were divided and incubated for 30 min at 4°C either without or in the presence of 50 ng/μl proteinase K, or with 50 ng/μl proteinase K and Nonidet P-40, respectively. The reaction was stopped with phenylmethylsulfonyl fluoride and proteins were separated by 10% SDS-PAGE. Proteins were visualized with the polyclonal rabbit antisera m06-2 in Western blot analysis.
radiation pathway is a common part of the ER quality control machinery. Despite the evidence that ER membrane export was independent of a functional proteasome (14, 15, 21, 42, 43), other reports indicated a direct involvement of the proteasome in the retranslocation event (19, 42, 44).

Here, we used the viral protein gp48 as a model system to get further insight into the mechanism of proteasome-dependent degradation of a glycoprotein, which is destined for degradation when it lacks its partner for assembly, the MHC class I molecule. The abundantly expressed glycoprotein gp48 of MCMV is a type I transmembrane protein, which binds to MHC class I molecules in the ER for transport through the Golgi and routing to lysosomal degradation (27). Unassembled excess gp48, however, is degraded by a pathway, which depends on a functional proteasome as demonstrated by proteasomal inhibitors and by reconstitution of microsomal membranes with purified proteasome.

Upon treatment of cells expressing gp48 with proteasomal inhibitors, the majority of the unassembled viral protein is neither rapidly transported into the cytosol nor accumulated in aggresome-like structures (25, 26). Instead this viral protein is sorted to the ER/ERGIC where the proteasomal degradation of the protein probably takes place. These findings are in accordance with earlier observations on other substrates, namely that unassembled MHC class I molecules destined for degradation accumulate in vesicles assigned to the ERGIC compartment rather than to the ER and co-localize with components of the ubiquitin-proteasome system (24). Therefore, we suggest that also the ERGIC can represent a compartment from which glycoproteins can be extracted and degraded. Whereas US2 and US11 directed degradation of the MHC class I heavy chain is a matter of minutes, the degradation kinetics of gp48 is slow and comparable with the CD3-δ subunit of the T cell antigen receptor, which takes about 2–3 h (42). Perhaps it is the dynamics of the process that defines the location at which a specific molecule is subjected to export and degradation.

As revealed by proteinase K digestion, gp48 remains correctly inserted into microsomal membranes upon inhibition of the proteasome. This suggests that a functional proteasome is involved in the transport of gp48 out of the membrane. For the degradation of the CD3-δ subunit of the T-cell receptor (42), the unassembled immunoglobulin light chain (44) and of Pdr" (45), a yeast protein (19), retranslocation is coupled to a functional proteasome. A short-lived synthetic model transmembrane protein required the proteasomal activity of the proteasome already for the dislocation process itself (40). This process was dependent upon the cytosolic tail of the target protein, which was marked for proteasomal degradation by ubiquitination. In agreement with this observation, the degradation of gp48 is tightly coupled to a functional proteasome. Different is the lack of a cytosolic tail and its ubiquitination as a signal for direct proteasomal interaction. We also failed to detect any other ubiquitinated forms of gp48 during proteasomal inhibition (data not shown). Therefore the example of gp48 demonstrates that a link between the proteasome and a membrane-inserted substrate must exist, which does not require a cytosolic tail.

In isolated microsomes containing gp48 the addition of ATP and proteasome reconstituted the degradation process. This confirmed the direct influence of the proteasome in the degradation of gp48 and there was no protein export from the microsomal membrane in the absence of a functional proteasome. Thus, both transport to the cytosol and degradation of the viral glycoprotein gp48 and gp48ΔCT is dependent on a functional proteasome.

Our data suggest that different cellular mechanisms for substrate delivery to subsequent proteasomal degradation must exist. First, from different cellular compartments the degradative process can be initiated. Furthermore, there are different extraction mechanisms for the proteins out of the membrane. For some viral glycoproteins the transport process is proteasome-independent (14, 15, 21). This could be due to the intrinsic properties of the viral proteins US2 and US11 to initiate substrate export. For other substrates the export can be proteasome dependent and mediated through the cytosolic tail of the protein (40). Since for gp48 the cytosolic tail of the substrate is not required, we propose that translocon-associated polypeptides connect in trans the substrate with the cytosolic proteasome. Along this line, Yu and Kopito (43, 45) showed that the proteasomal degradation of T cell antigen receptor α is dependent on a functional ubiquitination system, although there was no evidence of ubiquitination of the substrate itself. They proposed that ubiquitin might have an indirect effect on one or more translocon-associated proteins, perhaps by recruiting proteasomes to the dislocation site. Interestingly, during degradation of the misfolded lumen protein α1-antitrypsin, the ubiquitination of the chaperone calnexin was induced during interaction with the lumen substrate (46). gp48 is also associated with calnexin (data not shown) but it remains open whether this chaperone connects between proteasomal activity and dislocation of gp48. Recent data of Bordallo and co-workers (47, 48) that in Saccharomyces cerevisiae the ER resident protein Hrd 1p/Der3p serves as a component programming the translocon for retrograde transport of ER proteins. Depending on the individual substrate tested, the ER-associated degradation was either dependent or independent of HRD genes (49). Thus examples for yeast and individual substrates in mammalian cells suggest collectively that also in higher eukaryotes an indirect connection between the proteasome and its substrate must exist.

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Proteasomal Degradation of a Viral Protein
The Glycoprotein gp48 of Murine Cytomegalovirus: PROTEASOME-DEPENDENT CYTOSOLIC DISLOCATION AND DEGRADATION
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