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Dislocation of Type I Membrane Proteins from the ER to the Cytosol Is Sensitive to Changes in Redox Potential

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Abstract. The human cytomegalovirus (HCMV) gene products US2 and US11 dislocate major histocompatibility class I heavy chains from the ER and target them for proteasomal degradation in the cytosol. The dislocation reaction is inhibited by agents that affect intracellular redox potential and/or free thiol status, such as diamide and N-ethylmaleimide. Subcellular fractionation experiments indicate that this inhibition occurs at the stage of discharge from the ER into the cytosol. The T cell receptor α (TCR α) chain is also degraded by a similar set of reactions, yet in a manner independent of virally encoded gene products. Diamide and N-ethylmaleimide likewise inhibit the dislocation of the full-length TCR α chain from the ER, as well as a truncated, mutant version of TCR α chain that lacks cysteine residues. Cytosolic destruction of glycosylated, ER-resident type I membrane proteins, therefore, requires maintenance of a proper redox potential for the initial step of removal of the substrate from the ER environment.

Key words: diamide • class I heavy chain • degradation • human cytomegalovirus • TCR α chain

Major histocompatibility complex (MHC)1 class I products play a central role in the immune response against viral infection through their ability to guide CD8+ T cells to the infected cell (57, 67). To elude the immune system, several viruses have evolved strategies to prevent the surface expression of MHC class I molecules (34, 65). One example is human cytomegalovirus (HCMV), which encodes the US2 and US11 gene products that are at least partially responsible for blocking surface expression of MHC class I molecules (26). In cells that express either US2 or US11, MHC class I heavy chains are rapidly dislocated from the ER into the cytosol, an ATP-dependent process suggested to involve the translocon (Sec61p complex) (63, 64). Once in the cytosol, the single N-linked glycan on the class I heavy chains is removed by N-glycanase, and the polypeptide backbone is degraded by the proteasome. Indeed, deglycosylated cytosolic forms of the MHC class I heavy chains are observed only in the presence of proteasome inhibitors (63, 64).

Although physical removal from the ER and degradation of glycosylated type I membrane proteins by the proteasome were described for HCMV US2– and US11–induced proteolysis of class I heavy chains, it is likely that this mode of destruction is more generally used by the cell for turnover of misfolded and abnormal proteins in the ER (10, 12, 28). The occurrence of ubiquitin-conjugated intermediates of misfolded cystic fibrosis transmembrane conductance regulator and the inhibition of its proteolysis by lactacystin first suggested involvement of a cytosolic destruction pathway for this multispanning membrane protein (25, 60). The degradation of secretory proteins in Saccharomyces cerevisiae, such as mutant “misfolded” carboxypeptidase Y (CPY*), prepro α factor (ppαF), and the human α1-protease inhibitor, occurs in the cytosol in a proteasome-dependent manner (19, 35, 62). The transfer of CPY* and ppαF from the ER into the cytosol may involve the Sec61p complex (41, 42). Thus far, the molecular mechanism of degradation of proteins that are cleared from the ER remains poorly defined.

The maintenance of proper redox potential is critical not only for protein folding in the ER (11, 58) but also for

1 Abbreviations used in this paper: CPY, carboxypeptidase Y; diamide, di-azenedicarboxylic acid bis(N,N-dimethylamide); HCMV, human cytomegalovirus; IAA, iodoacetic acid; IAM, iodoacetamide; IEF, isoelectric focusing; MHC, major histocompatibility complex; NEM, N-ethylmaleimide; PDI, protein disulfide isomerase; TCR, T cell receptor; ZLH, carboxylbenzyl-leucyl-leucyl-leucinal; ZLVS, carboxylbenzyl-leucyl-leucyl-leucyl vinylsulfone.
class I heavy chains in US2/US11~ cells is affected when a more oxidizing environment is imposed by the addition of diamide or the alkylating agent N-ethylmaleimide (NEM). We show that the removal of type I membrane proteins from the ER critically depends on redox potential and is largely abolished by even modest concentrations of diamide and NEM.

### Materials and Methods

#### Materials and Inhibitors

Diamide, iodoacetic acid (IAA), iodoacetamide (IAM), and NEM were purchased from Sigma Chemical Co. (St. Louis, MO). The proteasome inhibitors carboxybenzyl-leucyl-leucyl-leucinal (ZL3H) and carboxybenzyl-leucyl-leucyl-leucyl vinylsulfone (ZL3VS) were synthesized as described (9, 63).

#### Cell Lines

U373-MG astrocytoma cells (control cells), US2 transfectants (US2~), and US11 transfectants (US11~) were prepared as described (26, 27). Daudi cells (American Type Culture Collection, Rockville, MD), which do not express β₂m (17, 37), were cultured in RPMI supplemented with 10% fetal calf serum. COS-1 cells were cultured in DME medium supplemented with 10% fetal calf serum.

#### Antibodies

Rabbit anti-class I heavy chain serum (αHC) (36) and the monoclonal antibody HC10 (52) recognize free class I heavy chains. W6/32 is a monoclonal antibody that recognizes assembled class I molecules (39). The rabbit anti-US2 serum was generated by immunizing rabbits with a fragment of US2 (amino acids 22–160 [13]) expressed in Escherichia coli. The polyclonal rabbit antisera R284 was raised against inclusion bodies of recombinant T cell receptor α (TR α) chain expressed in Escherichia coli (22). The anti-human transferrin receptor antibody (αTIR) is a monoclonal antibody (66Ig10) (59).

#### cDNA and Transfection

The cDNA of TCR α chain (HA 1.7) (18) and a truncated and cysteine-free form of TCR α (αVTM 3) chain was subcloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA), and liposome-mediated transfection was performed as described (22).

#### Gel Electrophoresis

SDS-PAGE, one-dimensional isoelectric focusing (IEF), and fluorography were performed as described (43).

#### Pulse-Chase Experiments

Cells were detached by trypsin treatment and then incubated with methionine- and cysteine-free DME with or without proteasome inhibitor ZL3H (25 μM) or ZL3VS (50 μM) for 1 h at 37°C. Cells were labeled by incubation with 400 μCi of [35S]methionine/cysteine (1,200 Ci/mmol; NEN-Dupont, Boston, MA) per milliliter at 37°C for the indicated times and chased with methionine- and cysteine-free DME supplemented with nonradioabeled methionine and cysteine to a final concentration of 2.5 and 0.5 mM at 37°C for the indicated times. Cell lysis and immunoprecipitation were performed as described (4). In experiments involving inclusion of diamide or NEM, they were added at the onset of the chase unless indicated otherwise.

#### Alkylation of MHC Class I Molecules in US2~ and Control Cells

A pulse-chase experiment was performed with US2~ and control cells in the presence of ZL3H (20 μM). The cells were pulsed for 3 min, chased for 3, 8, and 30 min, and lysed in a 0.5% NP-40 lysis mix containing iodoacetamide (10 mM) or iodoacetic acid (10 mM). Class I molecules were immunoprecipitated with either αHC or W6/32. A 3-min chase sample from

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The Journal of Cell Biology, Volume 142, 1998 366

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Figure 1. (A) Diamide induces the formation of disulfide bonds (i.e., reduced glutathione [GS~] is converted to its oxidized form [GSSG]). (B) NEM irreversibly alkylates free sulfhydryls (~SR).
control cells was immunoprecipitated with αHC and digested with bacterial N-glycanase (PNG; Boehringer Mannheim GmbH, Mannheim Germany) before loading onto the gel. The proteins were resolved by one-dimensional IEF or SDS-polyacrylamide gel (12.5%).

**Infection of Control Cells with a Recombinant Vaccinia Virus Expressing a Truncated Form of HCMV US2 (US2-150)**

Recombinant vaccinia virus (vvUS2-150) expressing a truncated form of HCMV US2 (amino acids 1–150) (US2-150) was a generous gift from Dr. John Yewdell (National Institutes of Health, Bethesda, MD). Control cells were infected with vvUS2-150 at a multiplicity of infection of 5 for 1 h in 50 μl of DME medium at 37°C. A 10-fold excess of DME medium supplemented with 10% fetal calf serum was added, and the infected cells were incubated for 3 h at 37°C. Cells were pulsed for 10 min with [35S]methionine and chased for 0 and 20 min as described above, except that 1 mM diamide was added to half of the cells after the 0-min chase point. Cells were lysed in 1% digitonin (wt/vol) in 25 mM Hepes, 150 mM potassium acetate, pH 7.7. Properly folded class I molecules and US2 were immunoprecipitated from the cell lysates using W6/32 and αUS2 antibodies, respectively (4). Precipitates were washed at 4°C with 0.2% digitonin in 25 mM Hepes, 150 mM potassium acetate, pH 7.7. Recovery of US2-150 from W6/32 precipitates was effectuated by incubating half of the W6/32 precipitates at 95°C for 5 min in the presence of 1% SDS followed by a 10-fold dilution with 0.5% NP-40 lysis buffer (4), and US2-150 was immunoprecipitated with αUS2 antibodies. The precipitates were then analyzed by SDS-PAGE.

**Subcellular Fractionation**

Approximately 5 × 10^6 US2^+^ cells were pulsed in 500 μCi [35S]methionine-cysteine/ml for 10 min. NEM (1 mM) or diamide (1 mM) was added 5 min into the pulse. Cells were chased for 0, 20, and 40 min and then homogenized by passing the cells (suspending in 250 mM sucrose, 10 mM Hepes, 150 mM potassium acetate, pH 7.7) 14 times through a ball bearing homogenizer (0.012-mm gap). Homogenized cells were spun (model TLA 100 ultracentrifuge) at 100,000 g for 1 h. The 100,000-g supernatant fraction was removed and adjusted to a final concentration of 0.5% NP-40, 125 mM sucrose. Unfractionated cells were lysised in 0.5% NP-40, 125 mM sucrose. All samples were subjected to immunoprecipitation using αHC, αUS2, or αTR.

**Degradation of a Transcated Form of TCR α Chain (V_aTM^4^) Lackying Cysteines**

V_aTM^4^, a truncated and cysteine-free form of TCR α chain (HA 1.7), was generated as follows: The constant domain of TCR α chain as well as Cys 209 were deleted by fusing the variable domain (Gln1-Pro121) in frame to the hinge region at Lys216. The remaining cysteines (Cys23 and Cys90) were changed to alanine by site-directed mutagenesis. The resulting homogenate was spun in a table top centrifuge (model 5415 C; Eppendorf Scientific, Madison, WI) at 1,000 g for 10 min, and then the supernatant was centrifuged for 1 h (as above) at 100,000 g. Pooled pellets of 1,000 and 100,000 g and the supernatant were resuspended in 0.5% NP-40 lysis buffer and subjected to immunoprecipitation with anti–TCR α chain serum. The precipitates were then analyzed by SDS-PAGE.

**Results**

**The Reduction of the Intrachain S-S Bonds of the Class I Heavy Chain in US2^+^ Cells Precedes Deglycosylation of the Heavy Chains**

The extracellular domain of MHC class I heavy chain contains four cysteines that form two intrachain disulfide bonds, one of which stabilizes the compactly folded α3 immunoglobulin domain. Cysteine residue 209 would be observed at the earliest time points of chase. However, if reduction precedes deglycosylation, the free SH content of class I heavy chains was monitored by the addition of either IAA, which resulted in the acquisition of a negative charge for each free SH modified, or IAM, which produces no charge difference (Fig. 2 A). Alkylated samples were analyzed by isoelectric focusing (IEF). The ratio of class I products expressed in most heterozygous cells lines results in heterogeneous banding patterns on IEF (Ref. 36, 53). The removal of the single N-linked glycan from the class I heavy chains results in conversion of Asn 86 to Asp, causing the heavy chains to acquire a negative charge (63, 64) (Fig. 2 B). The free SH content of class I heavy chains was monitored by the addition of either IAA, which results in the acquisition of a negative charge for each free SH modified, or IAM, which produces no charge difference (Fig. 2 A). A similar result is obtained when class I heavy chains from control cells were digested with N-glycanase in vitro (63) (Fig. 2 B, lane 1). If N-glycanase digestion were to precede the reduction of disulfide bonds, a comparison of samples treated with IAA and IAM should reveal the presence of alkylated, deglycosylated (Asp-containing) intermediates at the earliest time points of chase. However, if reduction precedes N-glycanase attack, then alkylated glycosylated class I heavy chains would be observed at the earliest time points of chase, to be followed by the appearance of the deglycosylated species.

Pulse-chase experiments were performed on US2^+^ cells in the presence of proteasome inhibitor, in conjunction with alklation using either IAA or IAM. Class I heavy chains were immunoprecipitated using a rabbit anti–heavy chain (αHC) serum, a reagent that reacts selectively with free heavy chains, or using the monoclonal antibody W6/32, which reacts with properly folded, β2m-associated heavy chains. The immunoprecipitates were then analyzed by
IEF or SDS-PAGE (43) (Fig. 2 B). In US2\(^+\) cells, at early time points of chase we detected the presence of more heavily alkylated species (Fig. 2 B, lanes 9 and 10, asterisks) that are absent from the control cells (Fig. 2 B, lane 15). At this time point, no deglycosylated intermediates were detected in the US2\(^+\) cells (Fig. 2 B, SDS-PAGE section, compare lanes 9 and 10). If the N-glycanase substrate is suppressed by prior treatment of cells with tunicamycin, a strategy used to avoid introduction of additional negative charges by cellular N-glycanase, we still observe increased representation of more heavily alkylated class I heavy chains in US2\(^+\) cells as compared with control cells (Fig. 2 B, lanes 12–15). We therefore conclude that the disulfide bridges of the class I heavy chains in US2\(^+\) cells are reduced before the removal of its N-linked glycan.

For completely folded class I molecules recovered with W6/32, there was no change in disulfide bonding status in either US2\(^+\) or control cells (Fig. 2 C), but there was a
steady decline in immunoreactive material in US2\(^{+}\) cells because of unfolding and attendant epitope loss (64). A charge shift was observed for folded, W6/32-reactive class I molecules in both control and US2\(^{+}\) cells upon alkylation with iodoacetate (Fig. 2C, compare lanes 1–5 and 6–10), which is almost certainly attributable to a free Cys in the cytoplasmic tail (45). The \(\beta_{2m}\) molecule does not contain any free cysteines, and therefore its isoelectric point does not shift upon alkylation with IAA (Fig. 2C, compare lanes 1 and 6). Given the kinetics with which reduction of intrachain -S-S- bonds and deglycosylation take place, pulse-chase experiments do not allow any further temporal resolution of these processes. Nonetheless, our data show that reduction of intrachain disulfide bonds precedes \(N\)-glycanase attack.

**Degradation of Class I Heavy Chains in US2\(^{+}\) Cells Is Inhibited by Diamide and NEM**

Free class I heavy chains occur in a reduced state before deglycosylation and probably dislocation in a US2-dependent manner (Fig. 2). Do dislocation of class I heavy chains from the ER and deglycosylation require reducing conditions? We shifted the redox potential of the cell towards a more oxidizing state by addition of either diamide or NEM and examined degradation of class I heavy chains in US2\(^{+}\) and US11\(^{+}\) cells. US2\(^{+}\) cells were pulse-labeled in the presence and absence of the proteasome inhibitor ZL3H (63, 64) and chased in the absence or presence of the indicated concentrations of diamide (Fig. 3). At 1 mM diamide, the degradation of free class I heavy chains was inhibited by 65% in the absence of the proteasome inhibitor (Fig. 3D). In the presence of the proteasome inhibitor and 1 mM diamide, 80% of the glycosylated class I heavy chains were recovered at the 25-min chase point compared with 30% of the glycosylated class I heavy chains recovered from untreated cells (Fig. 3). The conversion of glycosylated class I heavy chains to the deglycosylated intermediate was significantly inhibited in diamide-treated US2\(^{+}\) cells and occurs within minutes of diamide addition (data not shown). Comparable findings were obtained for cells expressing US11 (Fig. 4, A and B).

In a similar set of experiments, we examined the effects of different concentrations of the alkylating agent NEM on US2-dependent degradation of class I heavy chains (Fig. 5). Inhibition of both degradation and conversion of glycosylated heavy chains to the intermediate was observed at concentrations of NEM as low as 0.5 mM (Fig. 5, A and D). Free class I heavy chains recovered from NEM-treated cells migrate more slowly on SDS-PAGE than their counterparts for untreated cells (Figs. 5 and 6), an effect we attribute to the formation of covalent adducts between NEM and free \(-SH\) groups on the class I heavy chains. We conclude that the degradation of the class I heavy chains in US2\(^{+}\) cells and conversion of heavy chain
into its deglycosylated intermediate is sensitive to changes in redox potential.

**The Effect of Diamide and NEM on Properly Folded Class I Molecules in US2⁺, US11⁺, and Control Cells**

Properly conformed MHC class I molecules are also targeted for degradation by US2 and US11, but to a lesser extent. 50% of W6/32-reactive class I heavy chains were degraded in US2⁺ cells over a 25-min period in the absence of proteasome inhibitor (Fig. 3 B). However, in the presence of the proteasome inhibitor, only 27% of the W6/32-reactive class I heavy chains were degraded. The addition of 2 mM diamide to US2⁺ cells inhibits degradation of W6/32-reactive class I molecules by 80% (Fig. 3 B). The effect of diamide on class I heavy chain breakdown was also examined in US11⁺ cells in the presence of proteasome inhibitor (Fig. 4). 80% of the properly folded class I heavy chains in US11⁺ cells are degraded within 30 min (Fig. 4). However, only 24% of the W6/32-reactive class I heavy chains were degraded when US11⁺ cells are treated with 1 mM diamide. In fact, because dislocation was blocked and assembly can continue in the presence of diamide (Fig. 6), a relative increase in W6/32-reactive class I molecules was seen in cells treated with diamide.

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**Figure 4.** Diamide inhibits the conversion of the glycosylated class I heavy chains to its deglycosylated intermediate in US11⁺ cells. US11⁺ cells treated with the proteasome inhibitor ZL₃VS were pulsed for 10 min and chased for 0 and 30 min. Various concentrations of diamide were added at the onset of the chase. Cell lysates were immunoprecipitated with either anti-class I heavy chains (αHC) or W6/32. The immunoprecipitates were resolved by SDS-PAGE (12.5%). The glycosylated class I heavy chains (+CHO) are recovered by αHC and W6/32, while the deglycosylated intermediate (−CHO) is recovered with only αHC. A quantitative analysis (B) of the effect of diamide on the recovery of the glycosylated free (αHC) and properly folded class I heavy chains (W6/32) in US11⁺ cells was performed as described in Fig. 3.

**Figure 5.** NEM suppresses the accumulation of the deglycosylated class I heavy chain intermediate in US2⁺ cells. US2⁺ cells treated with or without the proteasome inhibitor ZL₃VS were pulsed for 10 min and chased for 0 and 30 min. Various concentrations of NEM were added at the onset of the chase as indicated. Cell lysates were immunoprecipitated with either anti-class I heavy chain (αHC) (A), W6/32 (B), or anti-US2 (αUS2) (C). The immunoprecipitates were analyzed by SDS-PAGE (12.5%). The glycosylated (+CHO) and deglycosylated (−CHO) forms of the class I heavy chains and US2 are indicated. A quantitative analysis (D) of the effect of NEM on the recovery of glycosylated free class I heavy chains using αHC in US2⁺ cells was performed as described in Fig. 3.
Similarly, NEM prevents degradation of W6/32-reactive class I heavy chains in a concentration-dependent fashion, with a maximum effect at 0.75 mM NEM (Fig. 5B). NEM treatment does not measurably alter the mobility of W6/32-reactive class I heavy chains, which is consistent with the notion that the heavy chains have already formed their intrachain disulfide bonds and contain at most a single free cysteine in the cytoplasmic tail.

Both diamide and NEM inhibit slightly the assembly of class I heavy chains into W6/32-reactive material (Fig. 6) when compared with untreated cells. Yet at the concentrations where dislocation and degradation were markedly affected, diamide and NEM do not block the folding of the class I heavy chains into properly folded class I molecules during the chase, as monitored by immunoprecipitation with W6/32 (Fig. 6).

The Effect of Diamide and NEM on the Degradation of the US2 Molecule

The amino acid sequence of US2 predicts it to be a membrane protein with three potential N-linked glycan attachment sites, only one of which is used (data not shown). The US2 protein recovered from US2+ cells exists in two forms, differing by the presence or absence of a single N-linked glycan. The nonglycosylated form of US2 was found in the cytosol (see Fig. 8) and was degraded in a proteasome-dependent manner (64) (Figs. 3C and 5C). This suggests that the US2 molecule escorts the class I heavy chains out of the ER lumen and into the cytosol, where both are degraded by the proteasome (64). In pulse-chase experiments of diamide-treated US2+ cells, the recovery of glycosylated US2 molecules was not affected (Fig. 3C). However, the amount of nonglycosylated US2 decreases significantly in diamide-treated cells (Fig. 3C) and parallels the decreased recovery of the deglycosylated intermediate for class I heavy chains. Similar results were obtained in pulse-chase experiments with US2+ cells in which NEM was added at the onset of the chase (Fig. 5C).

In diamide-treated US2+ cells, there is a reduced recovery of US2 associated with properly folded class I molecules (W6/32-reactive material) (Fig. 3B). The loss of the class I–US2 interaction in diamide-treated cells may be a direct consequence of diamide treatment. However, the interaction between the NH2-terminal 150 residues of
US2 and class I molecules is unaffected by diamide treatment, which suggests that the interaction between class I and US2 may involve other proteins of the degradation machinery. A recombinant vaccinia virus that drives the expression of US2-150 was used to infect control cells. Immunoprecipitation of class I molecules with W6/32 allows the coprecipitation of class I heavy chains with the US2-150 molecule (Fig. 7). Inclusion of diamide in the chase mix did not affect recovery of US2-150 by coprecipitation via the class I molecules. We conclude that the interaction of US2 with class I molecules is itself insensitive to inclusion of diamide.

**Diamide and NEM Block Dislocation**

Subcellular fractionation of lysates obtained from pulse-chased US2\(^+\) cells reveals the progressive release of class I free heavy chains into the 100,000-g supernatant fraction (cytosol) (Fig. 8). Absent from the cytosol fraction is the membrane protein transferrin receptor, which demonstrates the lack of membrane contamination in this fraction. Addition of diamide or NEM at 5 min into the 10-min pulse leads to an almost complete block in class I heavy chain dislocation to the cytosol. A small amount of the glycosylated class I heavy chains was recovered from the cytosol in diamide-treated US2\(^+\) cells. It is therefore possible that diamide may also inhibit N-glycanase activity. The presence of carbohydrate-bearing class I heavy chains in the cytosol (100,000-g supernatant) from diamide-treated cells suggests that complete dislocation can occur before N-linked glycan removal. The decrease of free class I heavy chains observed in unfractinated diamide-treated cells is accounted for by an increase in properly folded, W6/32-reactive molecules (Figs. 3 and 4). In the absence of proteasome inhibitor, glycosylated class I heavy chains do not accumulate in the cytosol, regardless of the presence of diamide (Fig. 8).

US2 molecules were recovered from subcellular fractions as described above (Fig. 8). The absence of nonglycosylated US2 molecules in the cytosol of fractionated US2\(^+\) cells treated with diamide or NEM is consistent with the lack of recovery of nonglycosylated US2 at later chase points in diamide and NEM-treated cells (Figs. 3 C, 5 C, and 8).

**The Effect of Diamide on the Degradation of Misfolded Glycosylated Membrane Proteins in the Absence of Viral Accessories**

The canonical example of degradation of a misfolded protein in the ER is the TCR \(\alpha\) chain (10). When the TCR \(\alpha\) chain is expressed in the absence of its normal molecular partners, the other subunits of the TCR–CD3 complex, it acquires a cytosolic disposition and is targeted for proteasomal proteolysis (22, 66). Since this process may use a dislocation mechanism similar to that seen for class I heavy chains in US2/US11\(^+\) cells, the fate of TCR \(\alpha\) chains in diamide-treated cells was examined. A pulse-chase experiment was performed on COS cells transiently transfected with TCR \(\alpha\) chain alone (Fig. 9 A). The TCR \(\alpha\) chains were immunoprecipitated using the conformation-independent polyclonal antiserum R284 (22). The degradation of fully glycosylated TCR \(\alpha\) chains was blocked in diamide-treated COS cells, both in the absence and presence of proteasome inhibitor (Fig. 9 A). However, recovery of partially deglycosylated breakdown intermediates at later chase points decreased in the presence of diamide. These results are analogous to those obtained for the recovery of deglycosylated class I heavy chains and nonglycosylated US2 molecules at later chase times (Fig. 3).

To determine the subcellular location of the TCR \(\alpha\) chains, homogenates of pulse-chased COS cells transfected with TCR \(\alpha\) chain were subjected to a centrifugation protocol that separates particulate and soluble fractions (see Materials and Methods) (Fig. 9 B). In diamide-treated cells, a small fraction of the fully glycosylated TCR \(\alpha\) chains was recovered in the soluble fraction, as observed for the class I heavy chains in US2\(^+\) cells (Fig. 8 A). The recovery of fully deglycosylated TCR \(\alpha\) chains in the soluble fraction of COS cells treated only with ZL3H is expected (22).
and its degradation is inhibited by the addition of diamide in a teasome-dependent manner as inferred from the sensitivity of the TCR to reduction (Fig. 10A). It contains one N-linked glycan and is recognized by the rabbit anti-TCR TM3H serum (Fig. 10A). This truncated TCR α fragment is expressed in a proteasome-dependent manner as inferred from the sensitivity of the TCR to reduction (Fig. 10B). This truncated TCR α fragment is expressed in a proteasome-dependent manner as inferred from the sensitivity of the TCR to reduction (Fig. 10B). This truncated TCR α fragment is destroyed in a proteasome-dependent manner as inferred from the sensitivity of the TCR to reduction (Fig. 10C). Since dislocation of the full-length TCR α chain is blocked by diamide, we suggest that diamide directly affects the free thiols within the dislocation machinery and not the substrate of dislocation. We conclude that a fragment of TCR α chain devoid of cysteines is destroyed in a diamide-sensitive manner.

Another example of a misfolded glycosylated membrane protein targeted for degradation is the MHC class I heavy chain in Daudi cells, which do not express β2m. Consequently, the class I heavy chains are unable to form properly folded complexes and are rapidly degraded (21, 40). The effects of diamide and NEM on the degradation of class I heavy chains in Daudi cells were examined in a pulse-chase experiment (Fig. 11). The addition of the proteasome inhibitor ZL3H to Daudi cells leads to the accumulation of a small amount of the deglycosylated breakdown intermediate at late chase points. The accumulation of the deglycosylated class I heavy chains at later chase points was also observed in US2+/US11+ cells. In the absence of the proteasome inhibitor, both diamide and NEM treatment of Daudi cells lead to a stabilization of class I heavy chains during the chase and the suppression of the deglycosylated intermediate. These results provide further evidence that membrane proteins targeted for degradation into the cytosol are sensitive to redox conditions.

**Discussion**

MHC class I heavy chains are targeted selectively for destruction by the HCMV gene products US2 and US11 (63, 64). This prevents surface expression of the MHC class I molecules, presumably allowing the virus to remain undetected by the immune system as long as the US2 and US11 genes are expressed. In studying this phenomenon, it has become apparent that the virus uses an unusual mechanism of destroying the class I molecule, a type I membrane protein. The proposed model of degradation of MHC class I heavy chains requires the cotranslational entry and glycosylation of heavy chains into the ER. The class I heavy chains are positioned either in the hydrophobic environment of the lipid bilayer or remain associated with the translocon, the Sec61p complex. They are then presumably extracted through the Sec61p complex into the cytosol. The role proposed of the Sec61p complex as the port of exit for proteins targeted for degradation in the cytosol is supported by genetic analysis in yeast. The degradation of the yeast mutant proteins CPY* and prepro α factor were retarded in yeast strains harboring mutant Sec61 alleles (41, 42).

HCMV seems to induce the degradation of class I heavy chains by a process that the cell would normally use to remove unwanted proteins from the ER. This entire process has been documented as ER-associated degradation. We refer to the step in which the type I membrane protein is exported from the ER as dislocation. The model protein that has been used in mammalian cells to study this type of degradation is the TCR α chain. Initially, TCR α chain was believed to be degraded in the ER itself, but more recent studies have shown that it is degraded in the cytosol by the proteasome (22, 66). Other misfolded membrane proteins that are targeted for degradation after the export from the ER are the cystic fibrosis transmembrane conductance regulator (25, 60) and the α subunit of the Sec61p complex.
in yeast (5). While a compelling case can now be made for cytosolic destruction of proteins that have been purged from the ER, very little is known about the molecular details of this series of reactions. Here we have focused on the role of the redox state in the ER as a variable that affects the dislocation reaction.

The reduction of the intrachain disulfide bonds of the class I heavy chains likely occurs before dislocation from the ER (Fig. 2), which is consistent with the notion that the heavy chains transported through the aqueous pore of the translocon are in an unfolded state. Even fully folded, W6/32-reactive molecules are destroyed in accelerated fashion when US2 or US11 is present (63, 64), and therefore the disulfide bonds already formed can be reduced. Because PDI is capable of mediating both oxidation of free SH groups, as well as reduction of S-S bonds already formed (15, 38), we consider it possible that folded class I molecules, while in an environment of high local PDI concentration as would presumably be found in proximity of the translocon, may be reduced by PDI.

Disrupting the redox potential of the cell by addition of diamide or NEM inhibits the dislocation of class I heavy chains from the ER to the cytosol in US2- and US11-expressing cells. In addition, diamide inhibits the degradation of a cysteine-free truncated form of the TCRα chain, the destruction of which is sensitive to inclusion of proteasome inhibitors. These observations suggest that the dislocation machinery must therefore contain thiols that are required for proper function. Both diamide and NEM interact with free −SH groups, as well as reduction of S-S bonds already formed (15, 38), we consider it possible that folded class I molecules, while in an environment of high local PDI concentration as would presumably be found in proximity of the translocon, may be reduced by PDI.

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the cytosol. Conversely, the recognition machinery of proteins targeted for degradation must exist within the ER lumen (50). Diamide-catalyzed oxidation of class I heavy chains does not explain why they are unable to dislocate, because similar results are observed with NEM, which simply alkylates free sulphydryls. The redox state of the free class I heavy chains recovered from NEM-treated US2+ cells is similar to that of heavy chains from untreated cells, yet dislocation is inhibited in NEM-treated cells. The interaction between class I heavy chains and US2 is not sensitive to inclusion of diamide, as inferred from the continued ability of a truncated form of US2 to interact with class I molecules in the presence of diamide. Hence, diamide must be affecting additional factors containing free thiols that are required for dislocation. An important advance in our understanding of the component parts of the machinery that supports vesicle trafficking has been the identification of a cytosolic, NEM-sensitive factor, or NSF (8, 16). Since 1 mM of NEM is required to inhibit NSF activity in an in vitro assay system, while treatment of intact US2+ cells with NEM shows an inhibitory effect on dislocation/degradation of class I heavy chains at 0.5 mM, we postulate the involvement of factors sensitive to NEM in dislocation. This property could perhaps be exploited for the purification of such factors, once an in vitro system capable of supporting US2/US11-dependent dislocation is available.

Notwithstanding overall inhibition of dislocation by diamide, small amounts of glycosylated class I heavy chains and a more substantial amount of fully glycosylated TCR α chains are found in the cytosol of diamide-treated cells (Figs. 9 and 10). Even though the different reports on the localization of mammalian N-glycanase are not consonant (55, 61), this observation suggests that glycosylated proteins can be dislocated and that prior deglycosylation of proteins is not required for dislocation, in agreement with our earlier suggestion (63, 64). In addition, N-glycanase activity has not been detected in Saccharomyces cerevisiae (46), yet cytosolic degradation of misfolded ER-resident proteins such as glycosylated CPY still occurs (19). Therefore, deglycosylation of ER proteins is not a prerequisite for dislocation.

Diamide also prevents the dislocation of the class I heavy chains from Daudi cells and the TCR α chain, which is consistent with the possibility that they are dislocated via a mechanism similar if not identical to that seen for US2/US11-catalyzed removal of class I molecules. Early studies of the degradation of misfolded ER proteins, such as TCR α chains, immunoglobulin light chains, and HMG-CoA reductase, show that diamide prevents their breakdown (2, 24, 51). Since these studies did not address the presence of breakdown intermediates in the cytosol, the degradation of these molecules was hypothesized to occur in the ER itself. The mechanism of action of diamide was believed to involve inactivation of an as yet unidentified cysteine protease in the ER lumen. Our data suggest that diamide interferes with degradation by blocking dislocation, rather than by inhibition of a —SH protease.

The luminal chaperones BiP (Kar2) and Sec63p are implicated in the degradation of mutant luminal yeast carboxypeptidase Y (42), but their role in the dislocation reaction is not understood in any mechanistic detail. However, both diamide and NEM affect cytosolic chaperone activity (31, 32). For example, yeast cells transfected with a plant cDNA of a protein whose amino-terminal end is homologous to the DnaJ family of chaperones provides protection to yeast cells treated with diamide (31). Since other members of the chaperone family aid the translocation of proteins across a membrane bilayer (14, 49), these findings suggest that cellular chaperones may be vital to the dislocation/degradation process of ER proteins and could constitute a target for NEM and diamide.

The cytosolic destruction of ER proteins is gaining acceptance as a more generally valid concept. A detailed understanding of this mechanism will provide insights into protein translocation across a membrane bilayer and help identify new factors that contribute to the degradation of misfolded and abnormal proteins.

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