Cutting Edge: Proteasome Involvement in the Degradation of Unassembled Ig Light Chains

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Several studies on disposal of nonsecreted Ig L chains have identified the endoplasmic reticulum as the site of degradation. Here, we examine degradation of a nonsecreted Ig L chain, T15L, and an experimentally endoplasmic reticulum-retained secretion-competent L chain, D16L, in the absence of H chains. We demonstrate that 1) degradation is specifically impaired by the proteasome-specific inhibitors carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone (Z-L3 VS) and lactacystin, 2) L chain degradation occurs early in the biosynthetic pathway, and 3) degradation does not require vesicular transport. Our findings indicate that previous assertions of L chain disposal within the endoplasmic reticulum must be modified. To our knowledge, we provide the first direct evidence supporting a new paradigm for removal of nonsecreted Ig L chains via dislocation to cytosolic proteasomes. The Journal of Immunology, 1999, 163: 11–14.

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The endoplasmic reticulum (ER) serves as the point of departure for properly folded and assembled cell surface and secretory proteins. Nascent polypeptides are subject to poorly understood quality control standards within this elaborate organelle; proteins that fail to fold or oligomerize correctly are, in most cases, retained and degraded without further progression along the export pathway. Recently, proteasome involvement in ER quality control of malfolded membrane-associated proteins (1–4), malfolded soluble secretory proteins (5–7), and nonmutant secretory proteins (8–10), but not Ig, has been demonstrated.

Ig variable regions exhibit extensive sequence diversity due to different combinations of germline genes and accumulation of somatic mutations. Igs are a natural model for exploring the influence of amino acid variation on protein assembly, secretion, and degradation. Quality control processes regulating Ig expression are complex and depend on isotype, assembly, and oxidation state of the Ig, as well as the maturation stage of the B cell (11–13). In a previous analysis of the negative effects of somatic mutation on Ig function, we found that 10% (16/160) of IgG2b transfectants with VH mutations were secretion impaired (14, 15). We examined the intracellular fate of four T15 Ab mutants and observed that the T15L chain, which is not secreted unless assembled with H chains, had two intracellular fates; most were degraded rapidly with a half-life of 1.3 h, whereas 5–20% of the L chain had a long half life paralleling the secretion-incompetent H chain (16). To begin to understand the differential mechanisms governing quality control of the T15L chain, we investigated the degradation of T15L chain expressed in SP2/0 myeloma cells in the absence of H chain.

We present evidence supporting the removal of nonsecreted Ig L chains via dislocation to cytosolic proteasomes. We show that the T15L chain localizes to the ER before degradation. Degradation of this secretion-impaired L chain is significantly decreased by proteasome-specific inhibitors, but not by inhibitors of vesicular transport or lysosome function. In addition, we also demonstrate that experimentally induced ER retention of a secretion-competent L chain leads to degradation via a pathway that is sensitive to proteasome-specific inhibitors, thus demonstrating proteasome involvement in L chain degradation.

Materials and Methods

Cell lines

SP2/0, SP2/0-T15L transfectant expressing the wild-type T15L chain and the D16H chain loss variant (D16H-) have been described (14, 17). PCM11 is an IgM hybridoma that is T15 Id positive (18) and expresses germline VH1 and Vx22 genes (G. Wiens, unpublished data), those used by T15. A PCM11 H chain loss variant was isolated as described (17). A T15L Thr→Asn replacement mutation was created by site-directed mutagenesis (Bio-Rad, Richmond, CA), and a stable SP2/0 transfectant was generated by electroporation (19) and maintained in G418. All cells were grown as described (14). Ig L chain content of lysates and supernatants was determined by ELISA as described (20). The percentage of intra- and extracellular Ig was calculated by comparing the amount detected in the supernatant or lysate with the total detected in the lysate plus supernatant.
Inhibitors
Carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone (Z-L,VS; a gift from M. Bogoy and H. Ploegh, Harvard Medical School, Boston, MA) was stored at −80°C in DMSO (16 mM). Lactacystin (Calbiochem, La Jolla, CA or Kamiya Biomedicals, Seattle, WA) was stored at 4°C in water (2.65 mM). Brefeldin A (BFA, Sigma-Aldrich) was dissolved in methanol (5 mg/ml) and stored at −20°C. Chloroquine (100 mM stock; Sigma-Aldrich, St. Louis, MO) was prepared in appropriate medium just before use.

Immunoreagents
Immunoreagents were used at the indicated dilution in PBS/10% FCS: polyclonal rabbit anti-recombinant human calreticulin (1:100); Affinity Bioreagents, Golden, CO); rabbit anti-mannosidase II (purchased from Kelly W. Moremen, University of Georgia, Athens, GA; 1:1000); biotinylated monoclonal rat anti-murine κ chain (PharMingen, San Diego, CA; 1:100); Texas red-X-conjugated goat anti-rabbit IgG H and L chain (Molecular Probes, Eugene, OR; 1:200); and FITC-streptavidin (Zymed Laboratories, San Francisco, CA; 1:200). Murine IgG2b (and IgAa (Zymed) were used in control reactions at 10 μg per 50 μL of diluted primary Ab solution.

Immunohistochemistry and confocal microscopy
SP2/0 or SP2/0-T15L cells, grown on coverslips, were washed with PBS, treated with 3% paraformaldehyde (4 mL/well) for 15 min, washed, and further incubated in PBS containing 10% FCS/0.5% Triton X-100 for 30 min. Coverslips were then inverted onto 50 μL of PBS/10% FCS containing the primary Ab and incubated for 2 h. Primary Ab was removed by washing with PBS, and the same procedure was used with the secondary immunoreagent(s). Washed coverslips were placed onto slides containing 15 μL of 1% n-propyl gallate (Sigma-Aldrich) in 1:1 glycerol/PBS, permanently mounted, and stored at 4°C in the dark. Confocal microscopy was performed with a Leica (Netzlar, Germany) confocal laser scanning microscopy and imaging system.

Biosynthetic labeling of cells and immunoprecipitation
SP2/0-T15L cells were grown in 24-well Primaria plates (Falcon/Becton Dickinson, Mountain View, CA) to 60–80% confluency. Cell monolayers were washed twice with DMEM lacking cysteine and methionine (Sigma-Aldrich) at 37°C, then incubated for 1 h at 37°C in deficient medium (1 ml per well). Cells were pulse labeled with 70 μCi of 35 S express labeling mix (NEN Life Sciences, Boston, MA) for 15 min, washed, and chased in IMDM, 20% FCS, for the times indicated (see Figs. 2 and 3). In experiments using proteasome inhibitors, Z-L,VS (16 μM) or lactacystin (25 μM) or an equivalent amount of diluent only (DMSO or H2O, respectively) was added during the preincubation period, as well as in the pulse and chase incubations. BFA or an equivalent amount of methanol was present during the pulse and chase periods. Chloroquine was present for the pulse and chase periods. For experiments employing D16H+ cells, trypsinized cells were plated at a density of 1 × 10^6 cells 24 h before the experiment. After washing, cells were labeled with 50 μCi of express labeling mix per well and chased for the times indicated (see Fig. 3). Supernatants were collected, and cells were lysed as described (21). κ L chains were immunoprecipitated with affinity purified polyclonal rabbit anti-κ (Cortex Biochemical, San Leandro, CA or ICN, Costa Mesa, CA) followed by protein A-Sepharose as described (16). Endoglycosidase H treatment of immunoprecipitated κ L chains was performed as directed (New England Biolabs, Beverly, MA).

SDS-PAGE and Western blotting
Protein A-Sepharose immunoprecipitates were resuspended in reducing SDS-PAGE sample buffer, and proteins were separated on either 10 or 12% acrylamide gels as described (16). Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad), and nonspécific sites were blocked with 0.05%–Tween-PBSA-1% BSA (fraction V; Calbiochem). κ-chain was detected by probing blots with a 1:5000 dilution of goat anti-mouse κ alkaline phosphatase conjugate (Southern Biotechnology Associates, Birmingham, AL). After extensive washing, immuneactive bands were detected using an Immun-Lite Chemiluminescent Substrate kit with the Immun-lite Enhancer (Bio-Rad) as directed.

Quantitation of immunoprecipitation
All labeling experiments were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and IP lab gel software (Version 1.5; Analytics, Vienna, VA).

Results and Discussion
Before degradation, the T15L chain is located in the ER
Ig L chain degradation is a well-studied example of ER-associated protein degradation, but the proteases involved and the cellular site of degradation have not been conclusively identified. Several previous studies demonstrated that nonsecreted L chain degradation takes place in a pre-Golgi compartment, suggested to be the ER (22, 23). We have shown that the T15L (Vκ22) chain is not secreted and is rapidly degraded if not paired with a secretion-competent H chain (16). To determine the subcellular location of T15L chains before degradation, we used immunofluorescent confocal microscopy. Immunofluorescent staining shows a significant overlap (Fig. 1A, panel 3) with anti-κ (panel 1) and the ER marker calreticulin (panel 2). Conversely, the L chain (panel 4) and the medial Golgi marker mannosidase II (panel 5) signals did not colocalize (panel 6). Anti-κ L chain Ab did not stain untransfected SP2/0 cells, whereas the anti-calreticulin and anti-mannosidase II
Abs produced staining patterns consistent with those observed with SP2/0-T15L cells (not shown). Staining by anti-k L chain Ab was completely blocked upon pretreatment of SP2/0-T15L cells with intact murine IgG2bc, but a similar pretreatment with IgAA had no effect (not shown). These results suggest that most or all T15L chain is located in the ER before degradation. Similar findings have been reported for CH12κ, another nonsecreted Ig L chain (22).

Although T15L appears to be predominantly or entirely confined to the ER, as judged by confocal microscopy, it was not possible to rule out accumulation of a small amount of T15L in an organelle beyond the ER before degradation. Therefore, a site-directed mutant containing a consensus N-glycosylation site, T15L T74N, was used to test whether any L chain progresses beyond the ER before degradation. Similar to the unmutated T15L chain, the T74N mutant was not secreted (Fig. 1B). The mutant L chain displayed slower electrophoretic mobility than wild-type L chain when the cells were cultured without tunicamycin, indicating that the consensus N-glycosylation site in the mutant had been utilized (Fig. 1C). In the presence of tunicamycin, the T74N mutant L chain migrated comparably to the wild-type T15L, which is not glycosylated. We found that T15L T74N remained endoglycosidase H sensitive, consistent with confinement to an early portion of the biosynthetic pathway (Fig. 1D). These results are in accord with the confocal results, indicating that T15L is located in the ER but not the Golgi and with the immunoprecipitation of T15 L chain with the ER chaperone H chain-binding protein (BiP) (not shown).

The rate of T15L degradation is decreased in the presence of specific, irreversible proteasome inhibitors

Previous studies on disposal of nonsecreted Ig L chains have suggested the ER as the site of degradation (22, 23). An investigation of the CH12κ chain showed that degradation required ATP and was sensitive to several serine protease inhibitors (22). Based on these findings, it was suggested that serine protease(s) within the ER is responsible for proteolysis of the CH12κ chain. In light of recent findings suggesting involvement of proteasome-mediated degradation in putative ER degradation, we investigated the possible role of this pathway in the clearance of unassembled Ig L chains. We began by examining the effects of treating SP2/0-T15L cells with two specific, irreversible inhibitors of proteasome activity, Z-L3 VS and lactacystin. A marked stabilization of the T15L chain was observed in cells treated with Z-L3 VS relative to untreated cells over the course of a 9-h chase (Fig. 2, upper panel). The t1/2 for degradation of T15L was 3.5 ± 0.8 h and 1.3 ± 0.5 h (n = 4, p < 0.05) in the presence and absence of the inhibitor, respectively. As an independent confirmation of this observation, a time course experiment was conducted using a structurally unrelated proteasome inhibitor, lactacystin (Fig. 2, lower panel). The t1/2 for degradation of T15L was 4.0 h and 0.6 h, in the presence and absence of lactacystin, respectively. Although the secretion of impaired mutant coagulation factor IX is at wild-type levels in the presence of high concentrations of proteasome inhibitors ALLM and ALLN (24), this was not true of T15L; there was no detectable L chain in tissue culture supernatants in any of the experiments (data not shown). Thus, increasing the intracellular load of L chains does not result in secretion, and cell lysis is minimal throughout the chase period.

To determine whether the degradation was peculiar to the SP2/0 T15L transfectant cell line, we also examined the effect of these proteasome inhibitors on the kinetics of T15L chain degradation in an H chain loss variant of the PCM11 hybridoma, which expresses an endogenous unmutated T15L chain. Similar to the SP2/0 T15L transfectant, in the absence of proteasome inhibitors, the t1/2 for the endogenous T15L chain was 1.5 h. Treatment with either lactacystin (25 μM) or Z-L3 VS (16 μM) extended the t1/2 to 4.0 h (data not shown).

T15L degradation is not affected by blockade of ER to Golgi vesicular transport nor by inhibition of lysosomal function

Degradation by proteasomes does not appear to be dependent on trafficking from the ER to the Golgi, nor is this process affected by inhibitors of lysosomal proteases (25, 26). Results from an earlier study indicated that the degradation of the nonsecreted CH12κ chain was not affected by agents that inhibit trafficking or lysosomal function (22). To confirm that degradation of the T15L chain is not dependent on trafficking from the ER to the Golgi or on lysosomal function, we investigated L chain degradation in the presence of BFA and chloroquine, respectively. We found that a concentration of BFA (5 μg/ml) sufficient to completely prevent secretion of Ig in SP2/0-T15L/T15H wild-type cells (data not shown) did not affect degradation of T15L (t1/2 = 1.16 ± 0.38 untreated vs 1.13 ± 0.33 BFA-treated, n = 6). Chloroquine (25 μM) also did not affect the rapid L chain degradation compared with untreated cells (t1/2 = 1.37 ± 0.51 untreated vs 2.15 ± 1.03 chloroquine-treated, n = 6). This concentration of chloroquine significantly inhibited degradation of total cellular protein (data not shown).

A secretion-competent L chain is also degraded by cytoplasmic proteasomes

To determine whether other IgL chains can be degraded by the proteasome pathway, we investigated the D16 H chain loss variant cell line, which efficiently secretes the D16L chain (Vκ1-C) (14). Pulse-chase experiments were performed using the ER to Golgi transport inhibitor BFA, the proteasome inhibitor Z-L3 VS, and both inhibitors. In the absence of inhibitor, secretion of D16L was complete by 4.5 h chase (Fig. 3A). Incubation with Z-L3 VS (16

![Figure 2](http://www.jimmunol.org/absabstracts/142/6/2341f2.jpg)
the proteasome inhibitor Z-L 3 VS (16) served almost complete persistence of a secretion competent some. Although the rates of T15L and D16L disposal were control standards as T15L and became substrates for the protea-
in complete inhibition of D16L chain secretion (Fig. 3 discussions; S. Stevens for expert technical assistance and for production of Enns research group (Oregon Health Sciences University) for many helpful
p persisting by 4.5 h chase (Fig. 3A/C), and a 6h period was observed, with only 15.0 ± 1.2% of the labeled L chain persisting by 4.5 h chase (p < 0.001 vs vehicle only). As expected, secretion was also completely prevented in the presence of both BFA and Z-L 3 VS. However, the rate of degradation of retained D16L chains decreased dramatically compared with the rate in cells treated with BFA only (Fig. 3D; 59.6 ± 17.9% remaining at 4.5 h, p < 0.05 vs BFA alone). These results suggest that ER-retained D16L chains are subject to the same or similar quality control standards as T15L and become substrates for the protea-
side of retained L chain longevity, they demonstrate that intracellu-

FIGURE 3. A secreted L chain is also degraded by cytoplasmic pro-
tesomes. The D16 H chain loss variant cells were metabolic labeled with 35S express labeling mix in the absence of inhibitors (A), in the presence of the proteasome inhibitor Z-L 3 VS (16 μM) (B), in the presence of BFA (5 μg/ml) (C), and in the presence of both inhibitors (D). Cells were chased for 0, 1.5, or 4.5 h. Autoradiograms shown are representative of two experiments each performed in triplicate.

To our knowledge these studies provide the first evidence that ER-retained Ig L chains are degraded by the proteasomal pathway. Although the data presented here support the involvement of cy-
tosolic proteasomes in clearance of L chains, we do not know whether the proteasome directly degrades the L chain or indirectly affects L chain stability, for example, by degrading a protease inhibitor. In a physiological setting, the ability to dispose of L chains that fail ER quality control via cytosolic proteasomes may re-

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