The *Listeria monocytogenes*-secreted p60 Protein Is an N-end Rule Substrate in the Cytosol of Infected Cells

**IMPLICATIONS FOR MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I ANTIGEN PROCESSING OF BACTERIAL PROTEINS**

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Cytosolic antigen degradation is an initial step in the generation of major histocompatibility complex (MHC) class I-associated cytolytic T lymphocyte epitopes. Intracellular *Listeria monocytogenes* secretes p60, a murein hydrolase, into the host cell cytosol, where it is degraded by proteasomes. Roughly 3% of degraded p60 gives rise to p60 217–225, a nonamer peptide that is bound by H-2Kb MHC class I molecules. Herein, we introduce targeted deletions throughout the p60 gene to identify potential proteolytic signals within p60. Degradation of mutant forms of p60 was investigated in macrophages infected with recombinant *L. monocytogenes*. We found that deletions within the amino-terminal two-thirds of p60 enhanced cytosolic degradation. In contrast, truncation of the C terminus resulted in modest stabilization of p60 in the host cell cytosol. Because a protein’s N-terminal amino acid can determine its rate of degradation, we mutagenized this residue in p60 into known stabilizing and destabilizing residues. Valine substitution dramatically stabilized cytosolic p60 molecules, while substitution with aspartic acid resulted in rapid degradation. The number of p60 217–225 epitopes isolated from infected cells directly correlated with the rates of p60 degradation. Our data, therefore, indicate that the N-terminal amino acid and multiple internal regions of p60 influence its stability in the cytosol of infected cells. Antigen degradation and epitope generation are linked, and different degradation signals can channel bacterial proteins into the MHC class I antigen processing pathway.

**MHC** class I molecules bind peptides that are generated in the cytosol by proteasome-mediated degradation of endogenous and foreign proteins (1–3). Proteasomes are multienzyme complexes consisting of a 20 S catalytic core that associates with regulatory subunits to make a 26 S particle. Membrane-permeable proteasome inhibitors prevent the generation of most antigenic peptides and, because of a shortage of peptide, impair MHC class I expression from the endoplasmic reticulum (4–6). The sites of proteasome cleavage can influence the efficiency of antigen processing into MHC class I-associated peptides (7–9). Two MHC-encoded proteasome subunit proteins, LMP-2 and LMP-7, and the proteasome regulator PA28 modify proteasome specificity and enhance MHC class I antigen processing (10–14). Peptides generated in the cytosol by proteasomes are translocated into the endoplasmic reticulum by the transporter associated with antigen processing (15) and, if they conform to the appropriate motif, are bound by MHC class I molecules (16). The assembled complexes travel to the cell surface, where they are presented to CD8+ cytolytic T lymphocytes (CTL). Cytosolic protein degradation is generally very specific and regulated. Thus, most endogenous proteins contain or acquire degradation signals prior to proteasome-mediated destruction. One of the best characterized mechanisms for marking proteins for cytosolic degradation involves the ubiquitination of target proteins (17). In this pathway, polyubiquitin chains are conjugated to one or more lysine residues of the target protein. Ubiquitin conjugation requires the action of multiple enzymes and is initiated by the recognition of specific protein sequences (17–19). Several signals that promote ubiquitination have been identified and include the N-terminal amino acid (19), internal sequences called “destruction boxes” (20), or even sequences on proteins associated with the degradation substrate (21). Ubiquitin-independent pathways for targeting intracellular protein degradation, possibly involving PEST sequences, have also been described (22–24).

Several lines of evidence suggest a role for the ubiquitin targeting pathway in MHC class I antigen processing. For example, cells with a defect in the E1 ubiquitin activating enzyme are incapable of processing microinjected chicken ovalbumin (25). Additionally, antigens can be targeted for MHC class I antigen processing by modification of the N-terminal amino acid into a destabilizing residue (26). While a correlation between antigen degradation and epitope generation has been demonstrated in multiple studies (6, 26, 27), there are conflicting reports demonstrating that protein degradation rates do not influence antigenicity (28, 29).

We have used macrophage cell lines infected with the intra-cellular bacterium *Listeria monocytogenes* as a model to investigate cytosolic antigen degradation and production of MHC class I-presented CTL epitopes. *L. monocytogenes* enters the cytosol of phagocytic cells by secreting listeriolysin O (LLO), which destroys the phagolysosomal membrane (30). Bacteria multiply intracellularly and secrete proteins that are processed into peptides that are presented by MHC class I molecules (31). In BALB/c (H2k) mice, LLO is processed into LLO 91–99 and p60, a murein hydrolase, is processed into p60 217–225 and p60...
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449–457 (32-34). These three peptides are bound by H2-Kb MHC class I molecules and are recognized by L. monocytogenes-specific CTL. Following secretion into the host cell cytosol, p60 is degraded with a half-life of approximately 90 min (35). Approximately 3% of degraded p60 molecules are processed into p60 217–225 (35), and approximately 30% are processed into p60 449–457 (34). LLO is also rapidly degraded in the host cytosol, and approximately 10–20% of degraded LLO molecules give rise to LLO 91–99 (36). Cytosolic degradation of LLO and p60 is proteasome-mediated (6), and there is a tight correlation between 1) the amount of antigen degraded and the number of CTL epitopes (35) and 2) the rate of antigen degradation and the rate of CTL epitope appearance (6).

It is unclear how p60 is targeted for degradation by host cell proteasomes. To determine if p60 contains a protein degradation signal recognized by the host cell, we made deletion mutants that spanned the majority of the mature protein and expressed them in L. monocytogenes. While most regions within p60 contribute to its stability in the host cell cytosol, the carboxyl-terminal region enhances degradation. Substitution of the N-terminal amino acid of p60 with stabilizing and destabilizing residues demonstrates that p60 is an N-end rule substrate. In agreement with our previous findings that degradation of p60 is tightly linked with epitope generation (6), we find a direct correlation between the rate of intracellular degradation of mutant forms of p60 and epitope generation.

EXPERIMENTAL PROCEDURES

Bacteria and Cell Lines—L. monocytogenes strain 43251 was obtained from the American Type Culture Collection (ATCC; Rockville, MD) and grown in brain heart infusion medium. Recombinant bacteria strains were grown in medium with spectinomycin (200 μg/ml). P815 mastocytoma cells (DBA/2, H-2d) and J774 macrophage-like cells (BALB/c, H-2d) were obtained from the ATCC and cultured in RPMI 1640 (Life Technologies) with 10% fetal calf serum, 2 mg/ml L-glutamine, 50 μg/ml 2-mercaptoethanol, 20 mM HEPES, penicillin, streptomycin, and gentamicin (RP10). CTL clone L9.6 is specific for p60 217–225 in the context of the H2-Kb molecule and was maintained by weekly restimulation with L. monocytogenes-infected J774 cells as described (33).

Peptide Generation and Presentations—Synthetic p60 217–225 (KYGVSVQDI) and p60 449–457 (AAGTGGACGGTGGACCATGCTCTG-3) were purchased from Geneset (Huntsville, AL). Peptides were HPLC-purified, and stock solutions were quantified by amino acid analysis. Ac-LLnL-CHO was purchased from Calbiochem and dissolved in Me2SO. Cycloheximide and anisomycin (Huntsville, AL). Peptides were HPLC-purified, and stock solutions were quantified by amino acid analysis. Ac-LLnL-CHO was purchased from Calbiochem and dissolved in Me2SO. Cycloheximide and anisomycin (Huntsville, AL).

Antigen Degradation and the Rate of CTL Epitope Appearance—L. monocytogenes were grown overnight to stationary phase. Cultures were centrifuged to remove bacteria and separated by SDS-PAGE (35). Proteins were transferred electrochemically to nitrocellulose, and membranes were blocked with milk, 0.1% Tween 20 and probed with anti-p60 rabbit antiseraum at a 1:4000 dilution (35). Blots were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin (IgG) in the secondary step and developed by enhanced chemiluminescence (Amersham, Buckinghamshire, United Kingdom).

Metabolic Labeling of Intracellular Bacteria and p60 Immunoprecipitation—L. monocytogenes-infected J774 cells were metabolically labeled as described previously (35). In short, 4 × 106 J774 cells were seeded in flasks and incubated overnight in RP10 medium with 100 μg/ml spectinomycin as the only antibiotic (RP-spc). Cells were infected with log phase cultures of recombinant L. monocytogenes (A600 = 0.1) for 30 min, washed, and incubated in RP-spc for 3 h. Gentamicin (50 μg/ml) was added after the first 30 min to inhibit extracellular bacterial growth. J774 cells were washed and placed in medium with 150 μM ‘methionine-free Dubecco’s modified Eagle’s medium with 3% dialyzed fetal calf serum, 2 mM L-glutamine, 20 mM HEPES, pH 7.5) with spectinomycin, gentamicin, the eukaryotic protein synthesis inhibitors cycloheximide (50 μg/ml) and anisomycin (30 μg/ml), and 25 μg/ml calpain inhibitor I (Llall), as indicated in the figure legends. After 30 min, translabel [35S]methionine at a specific activity of 200–500 μCi was added, and the cells were pulsed for periods varying from 20 min to 1 h. Cells were washed and chased for the time intervals indicated in the figure legends. After 10–20 min, cells were harvested in 1% Triton X-100 lysis buffer with protease inhibitors, and the detergents lysates were cleared by centrifugation (8 min at 14,000 rpm) at 4 °C. p60 was immunoprecipitated with 25 μl of protein A-Sepharose (50% slurry) and 5 μl of anti-p60 antiseraum for 1 h at 4 °C. Beads were washed four times and resuspended in sample buffer, and the samples were electrophoresed on SDS-10% polyacrylamide gels under reducing conditions. Gels were enhanced with 0.5 μl salicylate, 3% glycerol, dried, and exposed for autoradiography. Radioactive signals were quantified using a Bio-Rad GS-250 Molecular Imager.

Metabolic Labeling of Extracellular Bacteria—Recombinant L. monocytogenes were grown to log phase in brain heart infusion medium with 200 μg/ml spectinomycin from a culture of L. monocytogenes isolated on agar from a single colony, washed, and resuspended in methionine-free medium. After 30 min at 37 °C, bacteria were pulse-labeled with 100 μCi of [35S]methionine for 20 min, washed, and chased for 0, 30, and 60 min in RP10 with tetracycline. p60 was immunoprecipitated from the pulse medium and chase medium as described above. Samples were separated by SDS-PAGE, and gels were exposed for autoradiography and PhosphorImager analysis.

Peptide Extractions—CTL epitopes were extracted and HPLC-purified as described previously (33). In short, J774 cells were grown to confluence in 150-cm2 plates in RP10 with spectinomycin added the night before infection. Cells were infected with log phase cultures of recombinant L. monocytogenes for 30 min. The medium was then replaced with RP10 containing 5 μg/ml gentamicin, and the cultures were incubated for 4 h and then harvested in phosphate-buffered saline and pelleted. Cell pellets were stored at −80 °C. To elute peptides, the cells were resuspended in 10 ml of 0.1% trfluoroacetic acid, Dounce-homogenized, and sonicated. Lysates were ultracentrifuged at 100,000 × g for 30 min, and the resulting supernatants were lyophilized. Protein pellets were resuspended in 0.1% trfluoroacetic acid, passed through Centri-10 membranes, and separated by reverse phase HPLC. Fractions were monitored for fluorescence, lyophilized, and resuspended in 200 μl of phosphate-buffered saline, and stored at −20 °C.

CTL Assays and Epitope Quantifications—CTL assays were performed as described previously (33). Briefly, 103 51Cr sodium chromate-labeled P815 target cells were incubated with 50 μl of the HPLC-separated peptides and assayed for recognition by p60 217–225–specific CTL clone L9.6 (33). Using our standard protocol, p60 217–225 and p60 217Ser–225 elute in distinct fractions (36). p60 peptide-containing frac-
tions were titrated on P815 target cells and assayed in triplicate along with standard concentrations of synthetic p60 217–225 and p60 217Ser–225. Molar amounts of peptide were determined, and epitope numbers per cell were calculated as described previously (33).

RESULTS

Generation of p60 Mutants and Secretion by L. monocytogenes—To investigate how L. monocytogenes p60 is targeted for cytosolic degradation and processing into MHC class I-associated peptides, p60 gene deletion mutants were constructed (Fig. 1). Contiguous sections of p60 were deleted by site-directed mutagenesis from a gene encoding p60 217Ser. We used p60 217Ser because p60 217–225 generated from this protein contains serine instead of the wild type lysine in position 217, which alters the HPLC elution time of the epitope without affecting its recognition by the CTL clone L9.6 (34). The p60 signal sequence (amino acids 1–27) was maintained in all p60 constructs to ensure bacterial secretion (Fig. 1B). The deleted and truncated p60 genes were cloned into pAT29, a plasmid that replicates in Gram-negative and positive bacteria, and then introduced into L. monocytogenes. The resulting recombinant strains express both wild type p60, encoded by the chromosomal gene copy, and a plasmid-encoded, mutated form of p60. All recombinant strains multiplied at rates comparable with that of wild type L. monocytogenes and had a normal morphology (not shown), indicating that mutant forms of p60 did not interfere with bacterial growth and septation. To determine the rate of mutant p60 secretion, the supernatants of transformed L. monocytogenes cultures were separated by SDS-PAGE and probed in a Western blot with a polyclonal p60-specific antiserum (Fig. 2). The rate of mutant p60 secretion varied depending on which region of p60 was deleted. The amount of secreted, mutant p60 was proportional to the amount remaining associated the bacterial pellets (results not shown). p60 mutants lacking the N-terminal region (∆28–45) or the p60 217–225 epitope encoding region were not secreted at detectable levels and therefore were not included in our

Fig. 1. L. monocytogenes p60 and the derived deletion mutants. A, residues 1–27 encode a signal sequence that is removed upon bacterial secretion (37). p60 contains two CTL epitopes from amino acids 217–225 and 449–457 that are presented by H2-K1 MHC class I molecules. B, p60 mutants were generated from the full-length p60 217Ser construct as described under “Experimental Procedures.” All mutant forms were preceded by a signal sequence.
J774 macrophage-like cells were infected with recombinant L. monocytogenes. Culture supernatants from stationary phase bacteria were separated by SDS-PAGE and probed with a polyclonal p60-specific rabbit antiserum. The supernatant from wild type (w.t.) L. monocytogenes is shown in the left lane, while the right lanes show supernatant from the various p60 recombinant strains. The intensely staining protein found in all lanes is wild type p60, encoded by the bacterial chromosome. The deleted forms of p60 are of lower molecular weight and, depending on the deletion, are present in varying amounts. Molecular weight markers are shown on the left.

analyses. Altogether, approximately 94% of the p60 protein was deleted in this panel of p60 mutants.

Degradation of Truncated p60 in the Host Cell Cytosol—Wild type p60 is degraded in the cytosol of infected cells with a half-life of approximately 90 min (35). To investigate the intracellular degradation of the deleted and truncated forms of p60, J774 macrophage-like cells were infected with recombinant L. monocytogenes. Infected cells were metabolically labeled and chased for increasing time intervals, and p60 was immunoprecipitated and subjected to SDS-PAGE, autoradiography, and PhosphorImager analysis. Confirming our previous results, we found that wild type p60 secreted by the recombinant L. monocytogenes strains was degraded in the cytosol with a half-life of 60–90 min (Fig. 3). In contrast, deleted p60 Δ138–179, Δ265–305, and Δ304–365, was degraded much more rapidly (Fig. 3, A–C). Further experiments established half-lives of 15–20 min for these p60 products (Fig. 3E, Table I). In marked contrast to deletions in the N terminus or midsection of p60, deletion of amino acids 358–484 of p60 (p60 Δ358–484) produced a modest increase in the stability of p60 in the host cell cytosol (Fig. 3D).

LLnL, a membrane-permeable inhibitor of proteasomes, inhibits the proteolysis of p60 in infected J774 cells (6). We found that degradation of each p60 mutant was abrogated by treatment with LLnL (Fig. 3E and results not shown), suggesting that their degradation is also proteasome-mediated. Since LLnL enhances the metabolic labeling of intracellular L. monocytogenes (36) and since LLnL-induced effects on cellular proteolysis are rapidly reversible (4), we decided to use this inhibitor to facilitate the detection and analysis of poorly secreted p60 mutants. When J774 cells were pulse-labeled in the presence of LLnL and chased in its absence, wild type p60 was degraded normally (results not shown). Similar analysis of J774 cells infected with L. monocytogenes strains p60 Δ45–90, p60 Δ91–141, p60Δ170–216, and p60 Δ226–266 revealed that they were rapidly degraded with half-lives of 15–50 min (Table I). In summary, we find that deletions throughout the N-terminal and middle regions of p60 increase the intracellular degradation rate of this antigen, while truncation of the C terminus is modestly stabilizing.

Generation and Secretion of p60 Mutants with a Modified N Terminal—Although most deletions in p60 affected its intracellular stability, none resulted in a markedly prolonged half-life. Since the N-terminal residue determines the degradation rate of certain proteins (17–19), we decided to mutate the N terminus of mature p60 and to examine the effect on degradation in the host cell cytosol. Like most secreted bacterial proteins, p60 contains a signal sequence that is cleaved upon secretion. The specificity of signal proteases is dictated by amino acids within the signal sequence, particularly amino acids in the −1- and −3-positions. The protein sequence downstream from the signal sequence cleavage site, with the exception of proline in the +1-position, does not appear to significantly influence signal sequence cleavage (38). In the case of p60, the signal sequence is 27 amino acids long, and mature p60 has the amino acid 28 serine at its N terminus (37, 39). We mutated the codon for amino acid 28 from serine to the stabilizing amino acids valine and methionine and to the destabilizing amino acids aspartic acid and arginine (Fig. 1B). These mutations were performed in full-length p60 and also in p60 Δ358–484, which can be discriminated from the wild type form by SDS-PAGE (Fig. 1B). To establish that these mutants were secreted, we pulse-labeled extracellularly grown recombinant bacteria and chased in medium with tetracycline, added to stop bacterial protein synthesis. p60 was immunoprecipitated from the culture supernatant at the time intervals specified in Fig. 4. Wild type p60 was detected in the pulse media but not in chase media, indicating that bacterial p60 synthesis and secretion are essentially concurrent. In contrast, secretion of C-terminal truncated p60s was slightly delayed, with 10% of p60 Δ358–484 and 30% of the p60 Val28Δ358–484 and p60 Met28Δ358–484 still being secreted during the first 30 min after pulse labeling. In the case of p60 Asp28Δ358–484, roughly 50% was secreted during the first 30-min chase period. p60 Arg28Δ358–484 (not shown) was secreted at levels too low for meaningful quantitation and could therefore not be used in further experiments.

Intracellular p60 Degradation Is Influenced by Its N Terminal—We next compared the intracellular stability of the p60 Val28Δ358–484, p60 Asp28Δ358–484, and p60 Met28Δ358–484 mutants with that of p60 Δ358–484 by pulse-chase analyses (Fig. 5). As shown previously in Fig. 3, we again found that the rate of p60 Δ358–484 degradation was slightly diminished in comparison with wild type p60 (Fig. 5A). In contrast, the amount of p60 Val28Δ358–484 continued to increase during the first 60 min of chase (Fig. 5B), probably resulting from delayed secretion of this mutant form. During later chase intervals, however, very little degradation was observed, indicating that p60 Val28Δ358–484 is remarkably stable in the host cell cytosol. The calculated half-life between 60 and 180 min of chase was 3 h, while, in comparison, full-length wild type p60 was degraded with a half-life of 90 min (Fig. 5B).

Like p60 ΔVal28, p60 Met28Δ358–484 accumulated intracellularly in the first hour after pulse labeling (Fig. 5D). Degradation of this mutant form during later chase periods was much faster, with a t1/2 of 84 min between 60 and 180 min of chase. Therefore, placement of a Met at the N terminus does not have the same stabilizing effect as a Val in this position. Although the initial accumulation of p60 Met28Δ358–484 during the early chase period may be explained by delayed secretion, an alternative possibility is that p60 Met28 undergoes time-dependent modification prior to degradation. For example, the 35S-labeled methionine 28 may be removed by cytosolic Met aminopeptidases (40), exposing the position 29 threonine, a destabilizing residue, at the N terminal of the protein.

To facilitate analysis of p60 Asp28Δ358–484, infected J774 cells were treated with LLnL during the pulse and initial 60-min chase period, to provide an opportunity for p60 Asp28Δ358–484 to be secreted into the host cell cytosol. p60 was then immunoprecipitated immediately and 15 and 45 min after the removal of LLnL. Fig. 5C demonstrates that p60 Asp28Δ358–484 was rapidly degraded, with an approximate half-life of 17 min.

Our experiments indicate that the identity of the N-terminal
amino acid of mature, C-terminal truncated p60 plays a significant role in its cytosolic stability. Whereas Asp 28 is clearly destabilizing, Met 28 and the wild type Ser 28 confer intermediate stabilities, while Val 28 is the most stabilizing residue.

Epitope Processing from p60 Mutants with Different Stabilities—Generation of p60 217–225 requires p60 degradation and is directly related to the intracellular p60 concentration (6, 35). To investigate whether the rate of mutant p60 degradation correlates with the rate of epitope production, J774 cells were infected with the indicated recombinant L. monocytogenes strains and pulse-labeled with [35S]methionine. Cells were chased for the specified time intervals, and p60 was immunoprecipitated and subjected to SDS-PAGE. In panels A–D, labeled cells were chased for 0, 90, and 180 min. In panel E, labeled cells were chased for 0, 15, 30, and 60 min to facilitate analysis of the more rapidly degraded mutant p60. Additionally, the sensitivity of p60 Δ304–365 degradation to the proteasome inhibitor LLnL is demonstrated. The molecular weights are indicated to the left of each gel.

Epitopes in targeting fractions were quantified by titration of the appropriate HPLC fractions and comparison with a standard curve obtained with precisely quantified synthetic p60 217–225 and p60 217Ser–225, as described previously (33). We found that p60 217–225 numbers were comparable for cells infected with the different recombinant strains (Table II), indicating similar degrees of infection and intracellular bacterial growth. To facilitate the comparison of epitope production from the different deleted forms of p60, the numbers of extracted p60 217Ser–225 epitopes were normalized to compensate for variations in p60 secretion (Table II). The largest numbers of p60 217Ser–225 epitopes were generated from p60 mutants with internal deletions, which correlates with their high rate of intracellular degradation. However, the large number of p60 217Ser–225 epitopes obtained from several of the constructs (Δ226–266, Δ265–305, Δ304–365, and especially Δ45–90) is too great to be accounted for by enhanced degradation alone. In these cases it is likely that the deletions increase the efficiency of epitope generation.

Changing the N-terminal amino acid of mature p60 dramatically affected the generation of p60 217Ser–225. Because of unknown secretion levels, it was not possible to compare the epitope yields from full-length p60 mutants in infected cells (Table II). However, nearly 1800 and 4400 p60 217Ser–225 epitopes were generated from p60 Val28 and p60 Met28, respec-

Table I

| p60 mutants   | Intracellular half-life (min) |
|---------------|-------------------------------|
| Wild type p60 | 60–90                         |
| p60 217Ser    | 60–90                         |
| Full-length   | ND                            |
| Δ45–90        | 23                            |
| Δ91–141       | 18                            |
| Δ138–178      | 22                            |
| Δ170–216      | 50                            |
| Δ226–266      | 15                            |
| Δ265–305      | 16                            |
| Δ304–365      | 15                            |
| Δ358–484      | 92–140                        |
| Val28p60Δ358–484 | 180                        |
| Asp28p60Δ358–484 | 17                        |
| Met28p60Δ358–484 | 84                        |

a Infected J774 cells were subjected to pulse-chase analysis (see legend to Fig. 3). p60 bands were quantified from the polyacrylamide gels using a PhosphorImager, and half-lives were determined by plotting percentages of remaining p60 against the chase time intervals.

b Not determinable, because this mutant cannot be distinguished from wild type p60.

c Pulse labeling was in the presence and chase in the absence of LLnL to enable half-life determination.

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tively, reflecting the relative rates of intracellular secretion and degradation of these mutants. In the case of the N-modified, Δ358–484 truncated forms of p60, it was possible to correlate epitope generation with antigen secretion and degradation. After correcting for quantitative differences in antigen secretion, we found that roughly 20-fold fewer epitopes were produced from slowly degraded p60 Val28Δ358–484 and 7-fold fewer epitopes from the intermediately degraded p60 Met28Δ358–484 than from p60 Asp28Δ358–484. Epitope production from p60Δ358–484 was relatively high in comparison with p60 Met28Δ358–484, perhaps reflecting more rapid secretion of the former antigen. Alternatively, the relatively rapid degradation of p60 Met28Δ358–484, as detected in pulse-chase analyses (Fig. 5D), may reflect principally removal of the N-terminal methionine (which will reduce the 35S signal by nearly 50% since there is only one other methionine and one cysteine in p60 Δ358–484) rather than complete degradation of the truncated p60. Taking these factors into account, our findings demonstrate the direct linkage between antigen degradation and CTL epitope generation.

**DISCUSSION**

Cytosolic antigen degradation is fundamental to the generation of most MHC class I-presented peptides. In this report we have used an intracellular bacterial model to investigate cytosolic targeting of antigens for degradation and the relationship between degradation and epitope generation. In our analysis, we deleted sequential regions of the secreted _L. monocytogenes_ p60 protein and determined the impact of these mutations on intracellular stability. Deletion of 40–60-amino acid-long stretches from the N-terminal and middle region markedly enhanced p60 degradation. One possible explanation for this finding is that these deletions impair the folding of p60 following bacterial secretion, in which case mutant p60 may have the appearance of a denatured protein. Denatured proteins have been shown in reticulocyte lysates to be good substrates for ubiquitination and subsequent degradation (41). It is noteworthy that most of these short lived p60 mutants have only low expression levels in _L. monocytogenes_, perhaps resulting from rapid intrabacterial degradation. Thus, the proteolytic pathways that degrade aberrant proteins in bacteria and eukaryotes may respond to similar signals.

Protein degradation in the cytosol of eukaryotic cells is very specific. One mechanism that determines the stability of cytosolic proteins involves the identity of the protein’s N-terminal amino acid and is referred to as the N-end rule. Destabilizing and stabilizing amino acids have been determined in yeast, bacteria, and mammalian cells (19). In mammalian reticulocyte extracts, aspartic acid, arginine, and serine are destabilizing residues, while valine and methionine are stabilizing (42). The same hierarchy exists in mouse L cells, with the exception that N-terminal Ser is a stabilizing rather than a destabilizing residue (43). While the N-end rule has been defined using model proteins as degradation substrates, the number of known physiological substrates remains very small (17, 19). Furthermore, the role of the N-end rule in antigen processing is controversial. While one report has shown that placement of a destabilizing residue at the N terminus of an antigen enhances the presentation of CTL epitopes (26), another report has dem-

**FIG. 5.** The N-terminal residue influences the rate of p60 Δ358–484 degradation. J774 cells were infected with recombinant bacteria for 3 h, pulse-labeled with [35S]methionine, and chased for the indicated time intervals. p60 was immunoprecipitated and subjected to SDS-PAGE (left panels). The amounts of full-length and truncated p60 were determined by PhosphorImager analysis, and the percentage of each of the remaining p60 forms was plotted (right panels). Because the secretion of p60 Asp28Δ358–484 is delayed, cells infected with bacteria expressing this form of p60 (C) were metabolically labeled and chased for 1 h in the presence of LLnL prior to the 0-, 15-, and 45-min chase periods indicated in the figure.
onstrated that epitopes are presented equivalently from rapidly degraded or stable antigens (29).

We took advantage of the fact that p60 secretion into the host cell cytosol is accompanied by cleavage of a signal peptide, thus exposing a new N terminus (37, 39). This allowed us to investigate the role to the N terminus of mature p60 on its degradation in the host cell cytosol. We mutagenized the wild type serine N terminus of mature p60 into aspartic acid, valine, and methionine. To fully demonstrate the impact of the N-terminal amino acid of mature p60 into aspartic acid, valine, and methionine. To fully demonstrate the impact of the N-terminal amino acid influence p60 stability in a fashion that, overall, is consistent with the N-end rule, as defined in previous studies on N-end rule substrates (26), which demonstrated linkage between antigen degradation and epitope generation and are in agreement with the work of Shastri's and co-workers (29), who showed that the efficiency of antigen presentation did not correlate with the rate of antigen degradation. However, our systems are rather different in that we are investigating epitope generation from antigen introduced into the cytosol, while they are investigat-

shavsky and co-workers (43), the observed differences may reflect a difference in the acetylation of N-terminal Ser under the varying conditions. Acetyl conjugation (which blocks recognition by the N-end rule pathway) may occur more readily onto the N-terminal Ser of an endogenously synthesized substrate in mouse L cells than in reticulocyte lysates. Although postsecretion modifications of the N-terminal amino acid of mutant forms of p60 may influence their subsequent degradation rates, our findings indicate that differences in the identity of the N-terminal amino acid influence p60 stability in a fashion that, overall, is consistent with the N-end rule, as defined in reticulocyte lysates and in L cells.

The rate of p60 degradation, as determined by the N-terminal amino acid, strongly influences the rate of CTL epitope generation. We previously described the relationship between intracellular antigen concentration, stability, and the efficiency of CTL epitope production (35). Significant change in the rate of antigen degradation, such as changing the half-life from 180 to 15 min, increases epitope production approximately 330%. We found that aspartic acid at the N terminus enhanced p60 degradation. A valine at the N terminus stabilized p60. These findings suggest that p60 is an N-end rule substrate. While methionine at the N terminus did not substantially alter the intracellular half-life of p60 in pulse-chase analyses (Fig. 4D) when compared with p60 with wild type serine in this position, the number of epitopes produced from either full-length or C-terminally truncated p60 Met was significantly diminished (Table II). This suggests that p60 Met is stabilized relative to p60 Ser (wild type) and that the rapid degradation we determine may reflect removal of methionine from the N terminus rather than complete, rapid degradation of the whole antigen. Remarkably, while aspartic acid at the N terminus led to very rapid degradation, with a half-life in the cytosol of 17 min, stabilization by N-terminal valine was only partial, with a prolongation of the half-life to 180 min. Thus, it is clear that alternative degradation signals exist for the recognition of p60.

Given the discrepancy in stabilizing effect of N-terminal Ser in mammalian reticulocyte lysates (42) versus in mouse L cells (43), it is interesting that in our system the stability of p60 Δ358–484 with Ser at the N terminus falls between mutants with N-terminal Val and Asp. As discussed earlier by Var-

### Table II

| p60 mutants | Secretion<sup>a</sup> | Epitopes/infected cell<sup>b</sup> | Normalized numbers<sup>c</sup> |
|-------------|-----------------------|----------------------------------|---------------------------------|
|             | %                     | p60 217–225                     | p60 217Ser–225                  |
| Wild type p60 | 2716 (501)            |                                  |                                 |
| p60 217Ser   | ND<sup>d</sup>        | 1236 (55)                       | 9391 (1049)                     |
| Val<sup>e</sup> | 1284 (338)            | 1798 (112)                      |                                 |
| Asp<sup>e</sup> | 1573 (168)            | 167 (49)                        |                                 |
| Met<sup>e</sup> | 1316 (200)            | 4441 (403)                      |                                 |
| Δ 45–90      | 4                     | 1758 (241)                      | 674 (21)                        | 16,850 |
| Δ91–141      | 5                     | 1180 (146)                      | 241 (104)                       | 4520  |
| Δ 138–175    | 6                     | 1975 (211)                      | 217 (63)                        | 3617  |
| Δ170–216     | 3                     | 843 (42)                        | 96 (42)                         | 3200  |
| Δ226–266     | 4                     | 1336 (272)                      | 385 (21)                        | 9625  |
| Δ265–305     | 13                    | 1541 (481)                      | 1038 (78)                       | 7955  |
| Δ304–365     | 80                    | 1284 (723)                      | 6421 (1003)                     | 8026  |
| Δ358–484     | 300<sup>f</sup>       | 1300 (97)                       | 6117 (250)                      | 2039  |
| Val<sup>e</sup> Δ358–484 | 300<sup>f</sup>    | 1753 (406)                      | 395 (27)                        | 132   |
| Asp<sup>e</sup> Δ358–484 | 300<sup>f</sup>   | 2032 (109)                      | 809 (87)                        | 2697  |
| Met<sup>e</sup> Δ358–484 | 300<sup>f</sup>   | 1676 (58)                       | 1079 (33)                       | 360   |

<sup>a</sup> The relative amount of mutant p60 is expressed as the percentage of wild type p60 secreted by recombinant L. monocytogenes in infected cells. These values were determined by metabolic labeling of infected cells in the presence of L-L-norleucine and separation of immunoprecipitated p60 by SDS-PAGE followed by quantitation by phosphor image analysis (see legend to Fig. 5).

<sup>b</sup> J774 cells were infected with recombinant bacteria for 6 h and then acid-precipitated. Eluted peptides were HPLC-separated, and fractions were tested for recognition of p60 217–225 (HPLC fractions 41 and 42) and p60 217Ser–225 (HPLC fractions 43 and 44) by specific CTL clone L9.6. Peptides in target fractions were quantified, and the mean epitope numbers per infected cell were calculated from triplicate experiments as described (33). S.D. values are shown in parentheses.

<sup>c</sup> In order to facilitate the comparison of the different p60 mutants, we corrected for their different rates of secretion by normalizing the p60 217Ser–225 epitope numbers. The numbers listed in the last column of this table are the calculated p60 217Ser–225 epitope numbers if mutant p60 secretion occurred at 100% the rate of wild type p60 secretion.

<sup>d</sup> ND, not determined (wild type p60 and full-length mutant forms cannot be separated by gel analysis).

<sup>e</sup> p60 secretion was quantified from the culture media of metabolically labeled extracellular bacteria (see Fig. 4).
ing epitope generation from antigen synthesized in the cytosol. Thus, as recently reviewed by Yewdell et al. (44), the majority of CTL epitopes in the latter case may be derived from defective ribosomal products rather than resulting from the actual degradation of the mature protein substrate. In contrast, our experimental system focuses on epitope generation from intact proteins.

Our study suggests that multiple proteolytic signals can target proteins into the MHC class I antigen processing pathway. In our analysis of the p60 deletions mutants, we found that epitopes were generated from all forms of p60 (Table II), regardless of the rate of degradation. The direct correlation between degradation rates and epitope production indicates that recognition and destruction of cytosolic p60 by the host cell proteolytic machinery is the limiting step in CTL epitope generation. Metabolic stability of pathogen-derived proteins therefore is an important factor influencing antigenicity. Indeed, the rate of bacterial antigen degradation may be a more important determinant of antigenicity than antigen prevalence.

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