Characterization of Murine Cathepsin W and Its Role in Cell-mediated Cytotoxicity*

Jennifer K. Ondr‡§ and Christine T. N. Pham‡§

From the Departments of §Internal Medicine and ¶Pathology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110

Received for publication, January 12, 2004, and in revised form, March 5, 2004
Published, JBC Papers in Press, April 15, 2004, DOI 10.1074/jbc.M400304200

Cathepsin W is a member of the papain-like family of cysteine proteases. In this report, we have isolated the cDNA for murine CtsW (mCtsW) from a splenocyte library. The deduced 371-amino-acid sequence shares 68% identity with human CtsW and includes the conserved catalytic triad cysteine, histidine, and asparagine found in all members of this family. In addition to the full-length form of mCtsW, we have isolated an alternatively spliced form of the mRNA that lacks a complete catalytic triad. An S1 nuclease protection assay and a Western blot analysis showed that mCtsW is mainly restricted to the CD8+ T cell and natural killer cell compartments. In addition, we confirmed that, like its human homologue, mCtsW is localized mainly to the endoplasmic reticulum and its expression is up-regulated upon activation. We also characterized the mCtsW locus using bacterial artificial chromosome clones. The gene consists of 10 coding exons and 9 introns spanning 3.2 kb. To elucidate the physiologic role of this protease, we generated mice deficient in mCtsW. Our data establish that mCtsW is not physiologic role of this protease, we generated mice deficient in mCtsW. Our data establish that mCtsW is not required for cytotoxic lymphocyte-induced target cell death in vitro. In addition, mCtsW deficiency does not alter the susceptibility of cytotoxic lymphocytes to suicide or fratricide after degranulation. Thus, mCtsW does not have a unique role in target cell apoptosis or cytotoxic cell survival in vitro.

Human cathepsin W (CtsW), also named Lymphopain, is a member of the papain family of cysteine proteases. CtsW is largely restricted to the cytotoxic effector cell compartment, namely cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (1–3). The gene for CtsW is tightly linked to another cysteine protease, cathepsin (Cts) F, on human chromosome 11q13 (4). The linkage and high degree of similarity between these two molecules (58% at the amino acid (aa) level) resulted in the description of the cathepsin F-like subgroup with features more related to cruzipain, the well characterized cysteine protease of the protozoa Trypanosoma cruzi (4, 5).

Expression of human CtsW is largely restricted to CD8+ T cells and NK cells and up-regulated by interleukin (IL)-2 (3). Although CD4+ T cells express CtsW transcripts, no protein was detected in these cells by Western blot analysis (3). Other hematopoietic cells, such as B cells and monocytes, do not contain a significant amount of CtsW mRNA (1, 2). CtsW expression in vivo has been examined in patients with inflammatory bowel disease (Crohn’s disease and ulcerative colitis) and autoimmune gastritis (7). In gastrointestinal samples taken from patients with autoimmune gastritis, CtsW is detected in up to 65% of CD45+ cells. In contrast, the number of CtsW-expressing cells in Crohn’s disease and ulcerative colitis is much lower (11 and 6%, respectively). However, the specific role of CtsW in these diseases remains largely undefined.

The unique endoplasmic reticulum (ER) localization of human CtsW was first suggested by studies using epitope-tagged CtsW expressed in HeLa and Cos-7 cells (8). These results were confirmed by direct immunofluorescence using human CtsW-specific antisera as well as subcellular fractionation of HeLa cells transfected with untagged CtsW (3). ER localization usually requires retention signals such as a KDEL motif (9), KDEL-like motifs (10), C-terminal cysteine residues (11, 12), basic dipeptides within KXXX motifs (13), or internal signal sequences (14), all of which are missing from the CtsW cDNA sequence. Whether the 21-aa insertion, the 8-aa C-terminal extension, or other mechanisms participate in the active retention of CtsW in the ER will require further elucidation. Nonetheless, given the restricted pattern of expression and the predominant ER subcellular localization, it is tempting to speculate that CtsW participates in the processing of CTL/NK cell-specific substrates and that its enzymatic activity regul-
lates cell-mediated cytotoxicity. In fact, a recent report detected decreased cytotoxic activity in a human NK cell line treated with CtsW cDNA antisense oligonucleotides (9). This was the first study to support the hypothesis that CtsW is involved in cytotoxic processes. In this report, we characterized the mCtsW genomic locus. In addition, we generated a mouse deficient in CtsW using gene-targeted mutagenesis to further elucidate the role of mCtsW in cell-mediated cytotoxicity.

EXPERIMENTAL PROCEDURES

Molecular Cloning and Sequencing of mCtsW cDNAs—A cDNA library of total mRNA from mouse spleenocytes was generated using random hexamer priming. The primer pair 5'-atgacactgactgcccacctc-3' (forward) and 5'-tcaggaggacagaggctgc-3' (reverse) was used to amplify by PCR double-stranded cDNAs. These primers were based on the mouse CtsW cDNA sequence, NCBi accession number NM_009985. Amplified products were subcloned into pCR2.1 (Invitrogen). Plasmids with appropriate insert lengths were sequenced on an Applied Biosystems model 373 automated sequencer, using vector-based external primers and cDNA-specific primers based on previously obtained sequences.

Molecular Cloning and Sequencing of Murine Genomic CtsW—Full-length genomic CtsW clones were obtained by screening a mouse (129/SvJ) bacterial artificial chromosome library (Genome Systems) with mCtsW cDNA. The clones were digested with multiple restriction enzymes and subcloned into BluescriptKS (Stratagene). A 13-kb EcoRI fragment was found to contain the entire mCtsW gene. The exon/intron boundaries were sequenced and defined using vector and cDNA-derived primers. The entire locus was subsequently sequenced using intron-derived primers. Searching the GenBank™ data base, we found another bacterial artificial chromosome clone (RP23-389K5) from mouse chromosome 19 that contains a sequence with complete identity to the mCtsW locus. This was subcloned into pCR2.1 (Invitrogen) and sequenced using intron-spanning primers. The entire locus was subsequently sequenced using intron-derived primers. Searching the GenBank™ data base, we found another bacterial artificial chromosome clone (RP23-389K5) from mouse chromosome 19 that contains a sequence with complete identity to the mCtsW locus.

RNA Preparation and S1 Nuclease Protection Assay—Total RNA from adult mouse tissues and activated lymphocytes was isolated using the previously described guanidinium thiocyanate miniprep protocol from adult mouse tissues and activated lymphocytes was isolated using the previously described guanidinium thiocyanate miniprep protocol. The probe was amplified by PCR using the primer pair 5'-atcaaacaccagcag-3' (forward) and 5'-tgactcttcatgagacccagt-3' (reverse). The 404-bp product was gel-purified and end-labeled with [32P]ATP using polynucleotide kinase (Invitrogen). The S1 nuclease protection assay was performed as described previously using 25 μg of total RNA hybridized with 100,000 cpm of labeled probe (15). A β-actin-specific probe was used for RNA loading control (16).

Splenocyte Activation—Activated T cells were generated by culturing mouse splenocytes with 5 μg/ml concanavalin A (ConA) and 50 μ/ml recombinant human IL-2 as described previously (15). Lymphokine-activated killer (LAK) cells were produced by culturing splenocytes in high dose IL-2 (1000 units/ml) for 5–7 days. One-way mixed lymphocyte reaction (MLR)-induced CTL were generated by stimulating C57BL/6 splenocytes for 5 days with irradiated (2000 radians) BALB/c splenocytes at a 1:1 ratio. Splenocytes from mice expressing the DO11.10 T cell receptor (TcR) transgene (a gift from Dr. Osami Kanagawa) were stimulated as described previously (17, 18) to generate activated CD4+ T cells for use in S1 nuclease protection assays. Splenocytes from mice expressing the DUC18 TCR transgene (a gift from Dr. Paul Allen) were stimulated as described previously (19, 20) to generate the activated CD8+ T cells used in an S1 nuclease protection assay.

NK Cell Lines—NK37.12 cells, derived from mice expressing SV40 T antigen under the human granulocyte H promoter (21), were maintained in RPMI 1640 supplemented with 2 mM l-glutamine, 100 μg/ml non-essential aa, 1 mM sodium pyruvate, 15 mM Hepes, 50 μM Zn-mercaptoethanol, 100 μ/ml penicillin, 100 μ/ml streptomycin sulfate, and 500 units/ml recombinant human IL-2.

Production of a mCtsW-specific Rabbit Polyclonal Antiserum—The putative proform of mCtsW (aa 18-371) was directionally subcloned into the XhoI and EcoRI restriction sites of the expression vector pTrchHis (Invitrogen) and transformed into Escherichia coli, BL21 codon plus (Stratagene). Purified inclusion bodies, obtained from bacteria induced with isopropyl-1-thio-β-D-galactopyranoside for 4 h, were solubilized in 8 M urea and separated by removing contaminating protein by SDS-PAGE (22). Emulsified gel fragments were used to immunize rabbits (Biosource International).

Western Blot Analysis—Homogenized lymphoid organs and activated splenocyte cultures were washed in phosphate-buffered saline and re-suspended at 5 × 106 cells/ml in lysis buffer (25 mM Hepes, pH 7.0, 150 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, and a mixture of protease inhibitors (Sigma)). Cells were left on ice for 1 h followed by three freeze/thaw cycles, and the lysate was cleared by centrifugation at 15,000 rpm at 4 °C for 10 min. Lysate was resolved by 8% SDS-PAGE, proteins gel under reducing conditions and transferred to nitrocellulose. Immunoblotting was performed using rabbit anti-mCtsW antisera, 9545, at a 1:5,000 dilution. Rabbit anti-human β-actin (Sigma) was used as a protein loading control. Antibody-protein complexes were visualized with peroxidase-labeled goat anti-rabbit IgG (Sigma) and West Femto maximum sensitivity substrate (Pierce). IL-2/ConA blasts were sorted using flow cytometry based on CD4 (20%) and CD8 (80%) expression for use in Western blots.

Polyinosinic-polycytidylic acid (polyIC) Activation of NK Cells—Rag2-/- mice were injected intraperitoneally with 200 μl of 1 mg/ml polyIC or phosphate-buffered saline alone. After 48 h, mice were sacrificed, and spleens were processed for cell lysate as described above.

Endoglycosidase H (EndoH) Treatment—Cell lysate was left untreated or treated with 2500 units of Endo H (New England Biolabs) according to the manufacturer’s recommendations. The different conditions were fractionated on SDS-PAGE gels as described above and blotted for mCtsW with the specific rabbit antisera.

Transfection of NK37.12 and Cos-7 Cell Lines—The primer pairs 5'-caccagtacagctgcca-3' (forward) and 5'-gggaggacagaggctgc-3' (reverse) and 5'-caccagtacagctgcca-3' (forward) and 5'-ttggacagatgttacaa-3' (reverse) were used to amplify full-length mCtsW and alternatively spliced mCtsW. The products were subcloned into pDNR/1DV5 His TOPO expression vector (Invitrogen) that encodes a C-terminal V5 and a His6 epitope. NK37.12 and Cos-7 cells were transfected with 50 μg of DNA by electroporation with a BTX EC 630 using the settings 150 V, 800 microfarads, and 700 ohms. The vector pcDNA 3.1 was used as a negative control. Transiently transfected cells were allowed to recover for 24 h before harvesting. Stably transfected cell lines were established by selection in G418 at 1 mg/ml (Fisher).

Immunofluorescence—NK37.12 and Cos-7 cell lines were stained with mouse anti-V5 epitope (Invitrogen) and rabbit anti-BIP (Stressgen) or rabbit anti-granzyme A (MA2) (23). Fluorescein isothiocyanate goat anti-mouse IgG (Sigma) or Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes) were used as secondary reagents. After staining, slides were mounted with Vectashield (Vector) and visualized on a Nikon Eclipse E400. Pictures were generated using Magnafire software.

Construction of the CtsW Targeting Vector—The 13-kb EcoRI fragment containing the complete mCtsW gene locus was used to generate

FIG. 1. A comparison of deduced aa sequences of murine and human CtsW with mice identity between the two sequences. Gaps are introduced to optimize alignment. Asterisks indicate the cysteine, histidine, and asparagine residues of the catalytic triad, highlighted in boldface characters. Potential sites for N-linked glycosylation sites are underlined.

| HUMAN  | 5'-atcaaacaccagcag-3' | 5'-tcaggaggacagaggctgc-3' |
|--------|-----------------------|--------------------------|
| MOUSE  | 5'-atcaaacaccagcag-3' | 5'-tcaggaggacagaggctgc-3' |

Downloaded from www.jbc.org by guest on July 18, 2018
the targeting construct. The 3-kb 5′-arm that contains mostly 5′ upstream sequence was amplified by PCR using the primer pair 5′-gagactagtctaagctacta-3′ (forward) and 5′-gagactagtctgtttgcttcccaatc-3′ (reverse) and subcloned into pCR2.1 (Invitrogen). A 4-kb XbaI fragment containing exons 3–10 and 2.6 kb of 3′ flanking sequence was subcloned into pCR2.1 (Invitrogen) to be used as the 3′-arm of the targeting vector. To assemble the targeting construct, a 1.8-kb EcoRI fragment containing the neomycin phosphotransferase gene (driven by the phosphoglycerate kinase 1 gene promoter and flanked by LoxP sites (LoxP/PGK-Neo)) was subcloned into the EcoRI site of pCR2.1 containing the 1116-bp cDNA encoding the Cathepsin W in Cell-mediated Cytotoxicity published previously using 51Cr- or 125Iudr-labeled TA3 and YAC-1 target cells (25). A flow cytometry-based assay was established using green fluorescent protein (GFP) expressing effector or target cells. CtsW/W−/− mice were crossed to a GFP transgenic mouse on the C57BL/6 background (C57BL/6-tgACTbEGFP)10sb/J, Jackson Laboratories) to generate GFP-expressing CtsW/W−/− and CtsW/W−/− mice. To obtain a pure population of activated CD8+ cells, the CtsW mutation was bred into mice expressing the 2C TCR transgene (tg), which encodes a TCR and 3′ arm targeting construct. The 3-kb 5′-arm that contains mostly 5′ upstream targeted to the 3′-targeting arm. A 500-bp XbaI fragment from the 5′-targeting arm served as an internal probe. Homologous recombination in G418-selected ES clones was confirmed by Southern blot analysis using both internal and external probes. ES clones (clones 96 and 97) with correctly targeted CtsW allele were microinjected into blastocysts from superovulated C57BL/6 females and transferred into pseudopregnant females. Chimeric males were bred to 129/SvJ and C57BL/6 females. Germline transmission of the disrupted CtsW allele was confirmed by Southern blot analysis. Heterozygous animals were intercrossed to generate mutant mice. CtsW heterozygotes were backcrossed to C57BL/6 or maintained on pure 129/SvJ strain.

In Vitro Lytic Assays—In vitro lytic assays were performed using two different procedures. Isootope release assays were performed as described previously using [35Cr]- or [125I]-labeled TA3 and YAC-1 target cells (25). A flow cytometry-based assay was established using green fluorescent protein (GFP) expressing effector or target cells. CtsW/W−/− mice were crossed to a GFP transgenic mouse on the C57BL/6 background (C57BL/6-tgACTbEGFP)10sb/J, Jackson Laboratories) to generate GFP-expressing CtsW/W−/− and CtsW/W−/− mice. To obtain a pure population of activated CD8+ cells, the CtsW mutation was bred into mice expressing the 2C TCR transgene (tg), which encodes a TCR and 3′ arm targeting construct. The 3-kb 5′-arm that contains mostly 5′ upstream targeted to the 3′-targeting arm. A 500-bp XbaI fragment from the 5′-targeting arm served as an internal probe. Homologous recombination in G418-selected ES clones was confirmed by Southern blot analysis using both internal and external probes. ES clones (clones 96 and 97) with correctly targeted CtsW allele were microinjected into blastocysts from superovulated C57BL/6 females and transferred into pseudopregnant females. Chimeric males were bred to 129/SvJ and C57BL/6 females. Germline transmission of the disrupted CtsW allele was confirmed by Southern blot analysis. Heterozygous animals were intercrossed to generate mutant mice. CtsW heterozygotes were backcrossed to C57BL/6 or maintained on pure 129/SvJ strain.

In Vitro Lytic Assays—In vitro lytic assays were performed using two different procedures. Isootope release assays were performed as described previously using [35Cr]- or [125I]-labeled TA3 and YAC-1 target cells (25). A flow cytometry-based assay was established using green fluorescent protein (GFP) expressing effector or target cells. CtsW/W−/− mice were crossed to a GFP transgenic mouse on the C57BL/6 background (C57BL/6-tgACTbEGFP)10sb/J, Jackson Laboratories) to generate GFP-expressing CtsW/W−/− and CtsW/W−/− mice. To obtain a pure population of activated CD8+ cells, the CtsW mutation was bred into mice expressing the 2C TCR transgene (tg), which encodes a TCR and 3′ arm targeting construct. The 3-kb 5′-arm that contains mostly 5′ upstream targeted to the 3′-targeting arm. A 500-bp XbaI fragment from the 5′-targeting arm served as an internal probe. Homologous recombination in G418-selected ES clones was confirmed by Southern blot analysis using both internal and external probes. ES clones (clones 96 and 97) with correctly targeted CtsW allele were microinjected into blastocysts from superovulated C57BL/6 females and transferred into pseudopregnant females. Chimeric males were bred to 129/SvJ and C57BL/6 females. Germline transmission of the disrupted CtsW allele was confirmed by Southern blot analysis. Heterozygous animals were intercrossed to generate mutant mice. CtsW heterozygotes were backcrossed to C57BL/6 or maintained on pure 129/SvJ strain.

RESULTS

Isolation and Analysis of mCtsW cDNA—Primers based on the published mCtsW cDNA sequence (NCBI accession number NM_009985) were used to clone the 1116-bp cDNA encoding
In mCtsW, Cys151, His289, and Asn326 comprise the conserved translated, encodes a form of mCtsW that lacks His 289 and mCtsWalt catalytic triad found in all cysteine proteases. In addition to the full-length form of mCtsW, the entire open reading frame of 1081-bp splice variant of mCtsW (Fig. 2). The mCtsWalt-deduced protein sequence also diverged from full-length mCtsW after aa Asn204. The mCtsWalt-deduced protein has a regulatory function remains to be elucidated. Whether has been reported that some N-terminal sequences of cathespin W in Cell-mediated Cytotoxicity

Cathepsin W mRNA Restricted Pattern of Expression—Detection of mCtsW and mCtsWalt mRNA was accomplished using S1 nuclelease protection assays. A unique S1 probe was designed to hybridize specifically with exon 7, which contains the alternatively spliced sequence. Hybridization of the probe to full-length mCtsW resulted in a protected fragment of 130 nucleotides (nt), whereas hybridization to the alternatively spliced region located on a separate exon in the mouse system (data not shown). However, we cannot rule out the possibility that an untranslated 5'-exon exists in the mouse. The exon/intron borders use consensus donor/acceptor sites and are conserved between mouse and human (Fig. 3). In addition, murine and human CtsW exon lengths are conserved throughout, suggesting that the genes are true orthologues.

**CtsW Protein Expression in Activated Lymphocytes**—To
IL-2/ConA blasts and LAK cells are in vitro activated lymphocytes generated by exposure to exogenous IL-2. To clarify whether mCtsW up-regulation in these cells is due to activation or exogenous IL-2 exposure, we generated CTL by a one-way mixed lymphocyte reaction. The H-2b versus H-2d MLR-induced CTL expressed higher levels of mCtsW than IL-2/ConA blasts (Fig. 5D). In addition, we examined the expression of mCtsW in naive and in vitro activated NK cells. Rag2−/− mice were injected intraperitoneally with polyIC to induce NK cell activation, and after 24 h, their spleens were harvested. PolyIC-treated Rag2−/− splenocytes expressed much higher levels of CtsW than did those of phosphate-buffered saline (PBS) or PolyIC-treated lysates from IL-2/ConA-activated splenocytes, and lysates from MLR-induced CTL were probed with the mCtsW-specific antiserum. β-actin served as a control for protein content and quality.

**Subcellular Localization of mCtsW**—Previous reports suggest that human CtsW resides mainly in the ER (3, 8). To determine the subcellular localization of mCtsW, we attempted to perform immunofluorescence using the mCtsW-specific rabbit polyclonal antiserum. However, due to strong cross-reactivity with unknown proteins, we were unable to confirm the mCtsW localization by immunofluorescence using this antiserum. To circumvent this technical problem, we expressed mCtsW in NK37.12 and Cos-7 cell lines and used to perform immunofluorescence using the mCtsW-specific rabbit polyclonal antiserum. The fact that these mCtsW-expressing cell lines are not cross-reactive may indicate differences in glycosylation. CtsW, splenocytes were activated with IL-2/ConA, and CD4+ and CD8+ activated cells were sorted into pure populations. Note that there was little to no mCtsW protein detected in CD4−/−/activated T cells. D, mCtsW up-regulation was also detected following physiological activation. Spleen lysates from Rag2−/− mice injected with either phosphate-buffered saline (PBS) or PolyIC, lysates from IL-2/ConA-activated splenocytes, and lysates from MLR-induced CTL were probed with the mCtsW-specific antiserum. β-actin served as a control for protein content and quality.

**Subcellular Localization of mCtsW**—Previous reports suggest that human CtsW resides mainly in the ER (3, 8). To determine the subcellular localization of mCtsW, we attempted to perform immunofluorescence using the mCtsW-specific rabbit polyclonal antiserum. However, due to strong cross-reactivity with unknown proteins, we were unable to confirm the mCtsW localization by immunofluorescence using this antiserum. To circumvent this technical problem, we expressed mCtsW in NK37.12 and Cos-7 cell lines and used to perform immunofluorescence using the mCtsW-specific rabbit polyclonal antiserum. The fact that these mCtsW-expressing cell lines are not cross-reactive may indicate differences in glycosylation. CtsW, splenocytes were activated with IL-2/ConA, and CD4+ and CD8+ activated cells were sorted into pure populations. Note that there was little to no mCtsW protein detected in CD4−/−/activated T cells. D, mCtsW up-regulation was also detected following physiological activation. Spleen lysates from Rag2−/− mice injected with either phosphate-buffered saline (PBS) or PolyIC, lysates from IL-2/ConA-activated splenocytes, and lysates from MLR-induced CTL were probed with the mCtsW-specific antiserum. β-actin served as a control for protein content and quality.

**Subcellular Localization of mCtsW**—Previous reports suggest that human CtsW resides mainly in the ER (3, 8). To determine the subcellular localization of mCtsW, we attempted to perform immunofluorescence using the mCtsW-specific rabbit polyclonal antiserum. However, due to strong cross-reactivity with unknown proteins, we were unable to confirm the mCtsW localization by immunofluorescence using this antiserum. To circumvent this technical problem, we expressed mCtsW in NK37.12 and Cos-7 cell lines and used to perform immunofluorescence using the mCtsW-specific rabbit polyclonal antiserum. The fact that these mCtsW-expressing cell lines are not cross-reactive may indicate differences in glycosylation. CtsW, splenocytes were activated with IL-2/ConA, and CD4+ and CD8+ activated cells were sorted into pure populations. Note that there was little to no mCtsW protein detected in CD4−/−/activated T cells. D, mCtsW up-regulation was also detected following physiological activation. Spleen lysates from Rag2−/− mice injected with either phosphate-buffered saline (PBS) or PolyIC, lysates from IL-2/ConA-activated splenocytes, and lysates from MLR-induced CTL were probed with the mCtsW-specific antiserum. β-actin served as a control for protein content and quality.

**Subcellular Localization of mCtsW**—Previous reports suggest that human CtsW resides mainly in the ER (3, 8). To determine the subcellular localization of mCtsW, we attempted to perform immunofluorescence using the mCtsW-specific rabbit polyclonal antiserum. However, due to strong cross-reactivity with unknown proteins, we were unable to confirm the mCtsW localization by immunofluorescence using this antiserum. To circumvent this technical problem, we expressed mCtsW in NK37.12 and Cos-7 cell lines and used to perform immunofluorescence using the mCtsW-specific rabbit polyclonal antiserum. The fact that these mCtsW-expressing cell lines are not cross-reactive may indicate differences in glycosylation. CtsW, splenocytes were activated with IL-2/ConA, and CD4+ and CD8+ activated cells were sorted into pure populations. Note that there was little to no mCtsW protein detected in CD4−/−/activated T cells. D, mCtsW up-regulation was also detected following physiological activation. Spleen lysates from Rag2−/− mice injected with either phosphate-buffered saline (PBS) or PolyIC, lysates from IL-2/ConA-activated splenocytes, and lysates from MLR-induced CTL were probed with the mCtsW-specific antiserum. β-actin served as a control for protein content and quality.

**Subcellular Localization of mCtsW**—Previous reports suggest that human CtsW resides mainly in the ER (3, 8). To determine the subcellular localization of mCtsW, we attempted to perform immunofluorescence using the mCtsW-specific rabbit polyclonal antiserum. However, due to strong cross-reactivity with unknown proteins, we were unable to confirm the mCtsW localization by immunofluorescence using this antiserum. To circumvent this technical problem, we expressed mCtsW in NK37.12 and Cos-7 cell lines and used to perform immunofluorescence using the mCtsW-specific rabbit polyclonal antiserum. The fact that these mCtsW-expressing cell lines are not cross-reactive may indicate differences in glycosylation. CtsW, splenocytes were activated with IL-2/ConA, and CD4+ and CD8+ activated cells were sorted into pure populations. Note that there was little to no mCtsW protein detected in CD4−/−/activated T cells. D, mCtsW up-regulation was also detected following physiological activation. Spleen lysates from Rag2−/− mice injected with either phosphate-buffered saline (PBS) or PolyIC, lysates from IL-2/ConA-activated splenocytes, and lysates from MLR-induced CTL were probed with the mCtsW-specific antiserum. β-actin served as a control for protein content and quality.

**Subcellular Localization of mCtsW**—Previous reports suggest that human CtsW resides mainly in the ER (3, 8). To determine the subcellular localization of mCtsW, we attempted to perform immunofluorescence using the mCtsW-specific rabbit polyclonal antiserum. However, due to strong cross-reactivity with unknown proteins, we were unable to confirm the mCtsW localization by immunofluorescence using this antiserum. To circumvent this technical problem, we expressed mCtsW in NK37.12 and Cos-7 cell lines and used to perform immunofluorescence using the mCtsW-specific rabbit polyclonal antiserum. The fact that these mCtsW-expressing cell lines are not cross-reactive may indicate differences in glycosylation. CtsW, splenocytes were activated with IL-2/ConA, and CD4+ and CD8+ activated cells were sorted into pure populations. Note that there was little to no mCtsW protein detected in CD4−/−/activated T cells. D, mCtsW up-regulation was also detected following physiological activation. Spleen lysates from Rag2−/− mice injected with either phosphate-buffered saline (PBS) or PolyIC, lysates from IL-2/ConA-activated splenocytes, and lysates from MLR-induced CTL were probed with the mCtsW-specific antiserum. β-actin served as a control for protein content and quality.

**Subcellular Localization of mCtsW**—Previous reports suggest that human CtsW resides mainly in the ER (3, 8). To determine the subcellular localization of mCtsW, we attempted to perform immunofluorescence using the mCtsW-specific rabbit polyclonal antiserum. However, due to strong cross-reactivity with unknown proteins, we were unable to confirm the mCtsW localization by immunofluorescence using this antiserum. To circumvent this technical problem, we expressed mCtsW in NK37.12 and Cos-7 cell lines and used to perform immunofluorescence using the mCtsW-specific rabbit polyclonal antiserum. The fact that these mCtsW-expressing cell lines are not cross-reactive may indicate differences in glycosylation. CtsW, splenocytes were activated with IL-2/ConA, and CD4+ and CD8+ activated cells were sorted into pure populations. Note that there was little to no mCtsW protein detected in CD4−/−/activated T cells. D, mCtsW up-regulation was also detected following physiological activation. Spleen lysates from Rag2−/− mice injected with either phosphate-buffered saline (PBS) or PolyIC, lysates from IL-2/ConA-activated splenocytes, and lysates from MLR-induced CTL were probed with the mCtsW-specific antiserum. β-actin served as a control for protein content and quality.
Localized mCtsW to a compartment exhibiting a lace-like staining pattern typical of the ER (Fig. 6A). The epitope-tagged mCtsW pattern localized with BiP (Fig. 6A), an ER resident protein. In contrast, mCtsW did not co-localize with granzyme A, a known lysosomal protein (Fig. 6B). To further confirm the ER localization of mCtsW, we tested the sensitivity of CtsW to EndoH. Treatment of IL-2/ConA blasts cell lysate with EndoH localized mCtsW to a compartment exhibiting a lace-like stain-

**FIG. 6.** Subcellular localization of mCtsW. A, Cos-7 and NK37.12 cells stably transfected with a V5 epitope-tagged mCtsW cDNA was probed with anti-V5 (green) and anti-BiP (red) antibodies. Note that V5 staining co-localized with BiP (yellow). B, NK37.12 cells expressing mCtsW were stained with anti-V5 (green) and anti-granzyme A (red) antibodies. There was no co-localization of V5 and GzmA staining. C, lysates from IL-2/ConA-activated cells were examined in the absence or presence of EndoH using mCtsW specific antiserum. Note the decrease in the mCtsW band from 52 to 37 kDa, whereas the nonspecific upper band (asterisk) remained constant.

localized mCtsW to a compartment exhibiting a lace-like staining pattern typical of the ER (Fig. 6A). The epitope-tagged mCtsW pattern localized with BiP (Fig. 6A), an ER resident protein. In contrast, mCtsW did not co-localize with granzyme A, a known lysosomal protein (Fig. 6B). To further confirm the ER localization of mCtsW, we tested the sensitivity of CtsW to EndoH. Treatment of IL-2/ConA blasts cell lysate with EndoH reduced the molecular mass of mCtsW from 52 to 37 kDa (Fig. 6C). The increased mobility of mCtsW after EndoH treatment suggests that it contains mainly mannose oligosaccharide N-linked glycosylations and has not acquired more complex oligosaccharides, thus confirming the ER localization. mCtsW was also expressed in NK37.12 cells, where it also co-localized with BiP (data not shown). Although mCtsW was not detected in primary cells with the mCtsW-specific antiserum, this result suggests that mCtsW can be translated into a stably expressed protein. Identical results from both the NK37.12 and Cos-7 cell lines indicate that the unique ER localization of mCtsW is not dependent on a cytotoxic cell-specific protein.

**Generation of CtsW-deficient Mice**—We generated mice deficient in CtsW using gene-targeted mutagenesis to elucidate the physiologic function of mCtsW. The targeting vector was designed to remove the ATG containing exon 1 through intron 2 of the mCtsW gene and replacing them with a LoxP-flanked PGK-Neo cassette (Fig. 7A). The linearized targeting vector was introduced into RW-4 ES cells by electroporation, and G418-resistant clones were screened by Southern blot (Fig. 7B). Of 144 clones selected, 2 were correctly targeted. Both clones were injected into blastocysts from superovulated C57BL/6 females to generate chimeras. The resulting 15 male chimeras were bred to C57BL/6 females, and germline transmission of the targeted mCtsW allele was confirmed by Southern blot analysis of tail DNA (Fig. 7C). DNA digested with EcoRI and hybridized with the external probe yielded a 13-kb wild-type fragment and a 9-kb fragment from the mutant mCtsW allele. PstI digestion and hybridization with the internal probe yielded a 9-kb wild-type fragment and a 4.5-kb fragment when the DNA encoded mutant mCtsW allele. Heterozygous mating of CtsW+/− mice confirmed that the CtsW−/− progeny were viable. The CtsW−/− mice were born with the expected Mendelian ratios with equal representation of the both sexes and were healthy and fertile. An S1 nuclease protection assay confirmed that CtsW mRNA was absent in activated lymphocytes derived from homozygous mutant mice (Fig. 7D). Western blot analysis also established that lysates of activated lymphocytes derived from CtsW−/− cells were devoid of mCtsW protein (Fig. 7E). CtsW−/− mice were backcrossed to the C57BL/6 strain for at least six generations. At N6, speed congenics confirmed that CtsW−/− backcrossed mice were 97% congenic with wild-type C57BL/6 (data not shown). CtsW null mutation did not alter the development of the lymphocyte compartment. CD4+ T cells, CD8+ T cells, NK cells, and B cells were found in thymi, lymph nodes, and spleens of CtsW−/+ and CtsW−/− mice in similar percentages (data not shown).

**CtsW in in Vitro Lytic Assays**—CtsW-restricted expression to cytotoxic lymphocytes suggests a function unique to and shared by activated CD8+ T cells and NK cells. The capacity to induce target cell apoptosis is unique to cytotoxic effector cells and known to be regulated by ubiquitously expressed proteases, such as cathepsin C (also known as dipeptidyl peptidase I), and by CTL- and NK cell-specific proteolytic enzymes, such as granzymes A and B (29, 30). To evaluate the ability of CtsW−/− cytotoxic cells to induce target cell apoptosis, standard lytic assays quantifying the release of 51Cr (a measure of cell mem-
brane permeability) or 125Iudr (a gauge of DNA fragmentation) were used. CTL generated in a one-way MLR from CtsW+/+ and CtsW+/− spleenocytes were equally capable of inducing apoptosis of TA3 target cells, as determined by both 51Cr and 125Iudr release (Fig. 8, A and B). Both wild-type and CtsW-deficient LAK cells caused equivalent apoptosis in YAC-1 target cells over a range of cell concentrations in the isotope release assays (Fig. 8, C and D). Therefore, neither CTL nor LAK cells from CtsW-deficient mice differed from wild type in their ability to induce target cell apoptosis (Fig. 8). These data establish that mCtsW does not have a unique role in target cell apoptosis induced by in vitro activated CD8+ T cells and NK cells.

**CtsW in Fratricide and Suicide**—To determine whether mCtsW plays a role in the protection of CTL in suicide (death of a CTL caused by its own cytotoxic machinery) or fratricide (death of a CTL caused by cytolytic materials released from a neighboring CTL), we established a flow cytometry-based *in vitro* cytotoxic assay. We took advantage of the ectopic expression of GFP in either effector or target cells that allows for the differentiation of effector and target cells by flow cytometry. To obtain a pure population of activated CD8+ T cells, CTL were generated by stimulating spleenocytes from CtsW+/+ or CtsW−/− transgenic mice expressing the 2C TCR with irradiated BALB/c (H-2d) spleenocytes. These CTL were used as effectors with varying numbers of GFP-P815 (H-2d) target cells. After 6 h of incubation, cells were harvested, stained with 7AAD (a marker of apoptotic cells) (31), and analyzed by flow cytometry. Survival of target cells was calculated from the number of 7AADlow of apoptotic cells) (31), and analyzed by flow cytometry. Survival of target cells was calculated from the number of 7AADlow cells when compared with input target cells incubated with culture medium alone (Fig. 9A). CTL target lysis efficiency in this assay correlated well with lytic assays using 51Cr or 125Iudr (Fig. 9B). To test whether mCtsW protects effector CTL from suicide upon target cell engagement, we performed an experiment in which the number of effector cells was held constant, whereas the number of GFP-P815 target cells was varied. In a 6-h killing assay, we saw no difference in the number of remaining live CTL and LAK cells (Fig. 9C). Thus, mCtsW does not play a role in effector cell suicide.

To determine whether mCtsW protects CTL against fratricide, we used a similar approach in which the survival of IL-2- and ConA-activated T cell blasts (CAB) incubated with alloreactive MLR-induced CTL was measured (Fig. 9D). GFP-CAB were generated from mice that ubiquitously expressed GFP (see “Experimental Procedures” for the generation of these mice). Incubation of CtsW+/+ and CtsW−/− CAB with varying numbers of CTL resulted in the same percentage of CAB cell survival (Fig. 9E). Taken together, these results suggest that mCtsW is not involved in CTL self-protection from either fratricide or suicide.

**DISCUSSION**

This is the first description and characterization of the mCtsW locus. The high degree of identity as well as the con-
The cathepsins are a group of proteolytic enzymes that include aspartic, serine, and cysteine proteases. The largest group of cathepsins belongs to the family of papain-like cysteine proteases. These proteases all contain an active site or catalytic triad consisting of a cysteine, a histidine, and an asparagine residue (32, 33). The papain-like cathepsins are often referred to as “lysosomal proteases” due to their common subcellular localization, although the proteolytic activity, tissue distribution, and physiologic function differ widely among members of this family. Although CtsW shares 58% aa identity with CtsF, its restricted pattern of expression and ER localization sets it apart from the rest of the members of the cathepsin family.

The fact that both murine and human CtsW expression are restricted to the CDS “ + ”T cells and NK cells suggests that this molecule is involved in a unique and conserved process common to the function of both types of cytotoxic effector cells. However, to date, no specific physiologic substrate or function has been described for CtsW. Substrate specificity of the papain-like family of proteases is defined by the S2 binding pocket. A structural model of CtsW predicts that this pocket is lined by several residues containing aromatic rings (Phe<sup>195</sup> and Phe<sup>203</sup>), suggesting a preference for substrates with Leu or Phe in the P2 position (6). Consistent with these predictions, Brown et al. (1) detected higher activity using the substrate Bz-Phe-Val-Arg-NHMec in cell-free translation products of human CtsW. However, this substrate specificity was not confirmed in similar studies performed by other investigators using recombinant human CtsW (3). It is possible that the enzymatic activity of CtsW requires different substrate specificity (i.e. amino- or carboxyl-peptidase activity) or the presence of a cofactor present in CTL and NK cells. Using total lysate from Cos-7 cells transfected with the full-length mCtsW, we detected a modest increase (1.5-fold over baseline) in activity using the universal cysteine protease substrate Z-Phe-Arg-pNA (data not shown).

However, given the fact that mCtsW was overexpressed in Cos-7 cells and the detected increase in activity was minimal, we could not definitively conclude that the cleavage of Z-Phe-Arg-pNA was due specifically to mCtsW. Moreover, in both activated splenocytes and NK cell lines, we consistently detected a 52-kDa band that should correspond to the inactivezymogen. Since our rabbit polyclonal antiserum was generated against the putative proform of mCtsW, it was unlikely that we failed to detect the mature form of this protein. EndoH exposure reduced the CtsW band to 37 kDa, the calculated molecular mass of the full-length sequence, further confirming that mCtsW remained largely unprocessed. In addition, previously published studies also failed to detect a smaller processed form of the protein, thus corroborating our interpretation (3, 7). Therefore, how CtsW functions as a protease will require further studies.

One of the more intriguing characteristic features of CtsW is its subcellular localization to the ER. In the absence of any specific ER retention signals, the mechanism that allows CtsW to remain in this compartment remains obscure. We determined that the ER localization of mCtsW does not require a CTL/NK cell-specific protein since Cos-7 cells transfected with mCtsW also expressed the protein predominantly in the ER compartment, as evidenced by the co-localization with the ER marker BiP. Co-immunoprecipitation studies also failed to identify proteins that might serve as anchoring partners for mCtsW in the ER compartment (data not shown). However, given its unique localization, it is tempting to postulate that CtsW may participate in some ER-associated regulatory functions.

The role of proteases in target cell apoptosis has been well documented. Some of these proteases, such as granzymes, have restricted expression, whereas others (caspases and cathepsin...
C) are more widely expressed. The restricted pattern of expression suggests that CtsW participates in cytotoxic effector cell function(s). To elucidate the physiologic role of mCtsW in target cell apoptosis, we generated mice deficient in CtsW. Although a recent publication showed reduced target cell lysis by a human NK cell line treated with antisense oligonucleotides (9), we established that mCtsW is not critical for in vitro CTL and NK cell function in the mouse, as measured by standard 51Cr and 125I-tdr release assays. Given its high degree of homology and its conserved expression pattern, it is unlikely that the function of this protease differs between species. However, it is possible that mice are redundant for CtsW. Similar redundancy in cytotoxic effector cell functions has been demonstrated with the serine proteases, granzymes A and B (34). Although the granzyme A- and granzyme B-deficient CTL had no or minimal phenotype, the granzyme A and B double deficient CTL displayed a marked defect in inducing target cell apoptosis (24, 34–36). In addition, we cannot rule out that mCtsW contributes to cytotoxic effector cell function in vivo, against specific targets. An example of specific in vivo CTL function can be seen with the granzyme A-deficient mice. Although granzyme A-deficient CTL have normal effector functions in vitro, granzyme A-deficient mice are uniquely more susceptible to in vivo infection with the virus ectromelia (37). Thus, we are currently exploring the role of mCtsW in the host defense against various viral infections. It is also possible that mCtsW plays a regulatory role in the function of cytotoxic lymphocytes. In addition to the granule exocytosis and the Fas/FasL pathways, cytotoxic lymphocytes also exert cytolytic function through the release of soluble cytokines, such as tumor necrosis factor and interferon γ, to name a few. Due to its unique ER localization, mCtsW may participate in the production and/or release of these inflammatory factors. We are also currently pursuing these lines of investigation.

Lastly, we assessed the role of mCtsW in CTL defense against the potentially damaging contents of their own granules and those of their neighbors. Recent studies suggest that cathepsin B may protect CTL from self-destruction after degradation by cleaving and inactivating the pore-forming molecule perforin (38). Because these studies were performed using peptide-based inhibitors, the authors could not rule out the possible role of other proteases in this CTL defense process. Using a sensitive flow cytometry-based in vitro assay, we established that mCtsW does not participate in the in vitro protection of CTL against suicide or fratricide.

In summary, we have established that despite its restricted expression in CTL and NK cells, CtsW does not participate in cell-mediated cytotoxicity in vitro. However, given its restricted pattern of expression, its unusual subcellular localization, and the high degree of conservation in structure/localization between human and mouse, CtsW likely plays a regulatory role in function(s) common to these two cellular compartments. The generation of CtsW-deficient mice provides a unique tool that will facilitate future studies aimed at dissecting the true cellular function of this protein.

Acknowledgments—We thank April Adkinson for her assistance in the initial cloning and characterization of mCtsW. We also thank Elaine Ross and Ron McCarthy for performing the embryonic stem cell work and the microinjections. We thank Hector Molina and Alec Cheng for many helpful discussions.
Characterization of Murine Cathepsin W and Its Role in Cell-mediated Cytotoxicity
Jennifer K. Ondr and Christine T. N. Pham

J. Biol. Chem. 2004, 279:27525-27533.
doi: 10.1074/jbc.M400304200 originally published online April 15, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400304200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 16 of which can be accessed free at http://www.jbc.org/content/279/26/27525.full.html#ref-list-1