ISOLATION AND SEQUENCE ANALYSIS OF SERINE PROTEASE cDNAs FROM MOUSE CYTOLYTIC T LYMPHOCYTES

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Cytolytic T lymphocytes (CTL)[1] and NK cells are well-characterized effector cells with potent cytolytic activity (1–4). How these killer cells damage their targets has not been resolved. Biochemical analysis performed in the last 3 yr with homogeneous populations of killer lymphocytes has already identified a pore-forming protein (PFP, perforin or cytolysin) (5–8) and several serine esterases (9–13) localized in the cytoplasmic granules of these cell types. More recent studies by Masson and Tschopp (14), and Tschopp and Jongeneel (15) described the identification and partial sequences of six different granule serine esterases (which they call granzymes) found in murine CTL. PFP lyses a variety of target cells, whereas the function of granule serine esterases is still unknown although it has been suggested that these enzymes and PFP may form a novel cytolytic cascade analogous to the humoral complement system (16, 17). Proteolytic enzymes have long been implicated in cytotoxic reactions, as implied from earlier studies with protease inhibitors (18–21). A more detailed structural analysis of serine esterases is clearly warranted to elucidate their functional role in cell-mediated killing.

An alternative strategy to direct purification and characterization of killer cell proteins, which has been used to study cell-mediated killing, has consisted of subtractive cDNA cloning. In this approach CTL-specific cDNA clones are generated first and then probed secondarily for their function. Notably, of the recently isolated CTL-specific cDNA clones, three code for distinct serine proteases, designated H factor (22), CCP1/CTLA1 (23, 24), and CCP2/granzyme C (23, 25). Recently, a modified differential screening procedure was developed in this laboratory to generate a broad representation of T lymphocyte subset-specific cDNA clones (26). Four CTL-specific

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[1] *Abbreviations used in this paper:* CTL, cytolytic T lymphocyte; DFP, diisopropylfluorophosphate; IPTG, isopropyl β-D-thiogalactopyranoside; LGL, large granular lymphocyte; PFP, pore-forming protein.

cDNA clones have been identified using this procedure and are shown here to code for serine proteases. One clone is identical to H factor, whereas the other three represent novel transcripts. One of these clones has been expressed as a fusion protein. Antibodies prepared against this fusion protein stain granules of CTL and react with two protein bands derived from granules.

Materials and Methods

Cells and Subcellular Fractionation. Methods for isolating and maintaining the cloned Th L2 and the cloned CTL L3 have been described before (27). L3 cells are Thy-1$^+$, LFA-1$^+$, Lyt-2$^+$, L3T4$^-$, and H-2L^d reactive. L2 cells are Mls^{a,d} reactive and are Thy-1$^+$, LFA-1$^+$, Lyt-2$^-$, and L3T4$^+$. To stimulate the cloned T cells, they were resuspended at 10^6–10^7 cells/ml and were cultured with Con A (Pharmacia Fine Chemicals, Piscataway, NJ) either at 10 μg/ml for L2 cells or 2 μg/ml for L3 cells. The specificities of CTLL-A11 and CTLL-R8 have been given elsewhere (28).

EL-4 (mouse thymoma), YAC-1 (mouse leukemia), A20.2j (mouse B cell line), and K46 (mouse B cell line) cells were maintained in RPMI 1640 medium containing 5% FCS. When indicated, EL-4 cells were treated with 12-0-tetradecanoylphorbol-13-acetate (10 ng/ml) for up to 20 h and the stimulation was monitored by IL-2 assay of EL-4 cell supernatant using IL-2-dependent CT6 cells. Murine melanoma cells, Cloudman S-91, were grown in Ham's F-10 medium containing 8% newborn calf serum, 8% NU-serum (Collaborative Research, Lexington, MA), 100 μg/ml penicillin, and 200 U/ml streptomycin.

Large granular lymphocytes (LGL) were obtained from murine spleen cells essentially as described (29). Briefly, splenic mononuclear cells, enriched by Ficoll/Hypaque gradient centrifugation, were subjected to adherence in plastic dishes and passed over nylon wool columns to remove monocytes/macrophages and B cells, respectively. Nylon wool-nonadherent cells were cultured in T flasks at 2×10^6 cells/ml in medium containing 1,000 U/ml of rIL-2 (a generous gift of Cetus Corp., Emeryville, CA). After 4 h of incubation at 37°C, adherent cells received separately fresh medium containing 1,000 U/ml of rIL-2. Cells were incubated for 3–5 d, after which they were harvested and used for immunofluorescence or immunoblotting analysis. In a parallel experiment, cells prepared this way were shown to be cytotoxic to YAC-1 targets and were stained positively (>90%) with anti-asialo GM-1 antibodies. The rat LGL used here consisted of RNK cells, kindly provided by Dr. P. Henkart (National Institutes of Health, Bethesda, MD).

MLRs were established by using DBA/2 and C57BL/6 splenocytes as stimulating cells and C57BL/6 and CBA/J spleen cells as responders. Stimulating cells were irradiated with 2,000 rad before mixing with responder cells at a ratio of 1:4. MLRs were carried out for 5–7 d at 10^6 cells/ml, after which cells were harvested and used for immunofluorescence.

Cell rupture was done by nitrogen cavitation as described (28). Nucleus-free cell lysates (2×10^7 cell equivalents/ml) were subjected to centrifugation at 13,000 *g* for 20 min in a Sorvall SS-34 centrifuge. The pellet was resuspended to the same original volume with a high-phosphate extraction buffer (28). Both the supernatant and the resuspended pellet were centrifuged at 350,000 *g* for 1 h using a table-top ultracentrifuge (model TL100; Beckman Instruments, Inc., Fullerton, CA) to sediment membranes. The supernatants were collected and used for immunoprecipitation. In some experiments, cells were biosynthetically labeled with [^{35}S]methionine at 1 mCi/10^7 cells/50 ml of methionine-free medium for 2 h, followed by incubation with 4% normal medium for an additional 12 h.

Construction of cDNA Libraries of CTLL-A11 and L3 Cells and Isolation of CTL-specific cDNA Clones. Cytoplasmic RNA of CTLL-A11 and Con A–stimulated L3 cells was extracted by a guanidine isothiocyanate-cesium chloride gradient centrifugation method (30). Poly(A)$^+$ mRNA of the L3 and CTLL-A11 cells was purified by chromatography on oligo-dT-cellulose column. dscDNA was synthesized from the poly(A)$^+$ mRNA. The cDNA was methylated at EcoRI sites to prevent cleavage, and Eco RI linkers were ligated to cDNA. After Eco RI cleavage, cDNA was enriched for molecules >250 kD by passage over 1 ml of Bio-Gel A-150m columns (Bio-Rad Laboratories, Richmond, CA). The cDNAs were inserted into the unique

Eco RI site of both λgt10 and λgt11 bacteriophage cloning vectors (31). We have previously isolated a group of cDNAs that are specific for T cells in contrast with B cells, using both positive and negative differential screening and RNA blot analysis of various lymphoid cells. The T cell-specific cDNAs were further studied to determine whether they were specific for cloned Th L2 or cloned CTL L3 cells and whether they were inducible by Con A or IL-2. A salient feature of the method was to analyze L2 or L3 cDNA species that failed to hybridize to sscDNA probes prepared from closely related cells, and thus allowed us to isolate T cell subset transcripts that exist at a low level (26).

RNA Blot Hybridization. Total cytoplasmic RNA (10 μg) or poly(A)$^+$ mRNA (10 μg) were fractionated on 1.2% agarose-formaldehyde gels and transferred to Gene Screen Plus (New England Nuclear, Boston, MA). Gel-purified inserts of cDNA were ^{32}P-labeled by nick translation and used as probes. Filters were prehybridized and hybridized at 42°C in 50% formamide, 5× SSC (1× SSC-150 mM NaCl, 15 mM sodium citrate, pH 7), 0.1% SDS, 250 μg/ml of salmon sperm DNA, and 10% dextran sulfate. Filters were washed at room temperature for 15 min in 2× SSC and 0.1% SDS, and at 42°C for 3 min in 0.1× SSC and 0.1% SDS several times. When a Northern blot was used multiple times for hybridization, the previous probe was removed by treating the membrane in 10 mM Tris-HCl (pH 7.0) and 0.2% SDS at 85°C for 1 h.

Genomic DNA Blot Analysis. High molecular weight DNA of mouse spleens was prepared as described previously (32). Restriction endonuclease digests of DNA were subjected to electrophoresis in 0.8% agarose gel at 4°C. The gel was denatured with 0.5 M NaOH/1 M NaCl and neutralized with 1 M Tris-HCl, pH 8.0, 1 M NaCl. The DNA was transferred to Gene Screen Plus as described by Southern (33). The blot hybridized with ^{32}P-labeled cDNA in a solution composed of 6× SSC, 5× Denhardt's solution, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA at 65°C for 18 h. The filters were then washed three times at room temperature for 10 min each in 2× SSC and 0.1% SDS, and two times at 65°C for 45 min each in 0.1× SSC and 0.1% SDS.

DNA Sequencing. DNA restriction fragments, subcloned in M13 vectors (34), were sequenced by the dideoxy chain termination technique (35), with modification made to accommodate 2'-deoxyadenosine 5'-[α-[^{35}S]thio]triphosphate (36). A forward primer (New England Biolabs, Beverly, MA) complementary to the lacZ sequence adjacent to the 5' side of the Eco RI site in λgt11 was used for the direct sequencing of cDNA insert end point in λgt11 (37).

Antibody Production, Affinity Purification, Immunofluorescence, and Immunoblotting. The λgt11 cDNA bearing L3-5 was plated onto *Escherichia coli* Y1089. Lysogens, selected essentially as described (38), were induced with 20 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 3–4 h. After three washes, bacteria were resuspended to OD_{600} of one in water. NP-40 was added to 1% and the bacterial suspension was lysed by freezing and thawing (3×) followed by extensive sonication using a probe. After sedimentation of the cell debris in a microfuge (10,000 *g*, 15 min), the supernatant was diluted threefold in PBS and applied to a Sepharose 4B column coupled with rabbit anti-β-galactosidase IgG (CooperBiomedical, Inc., Malvern, PA) at a load of 10^9 bacteria equivalents to 1 mg of coupled antibody. The column was washed with two cycles of alternate washes of PBS, PBS/0.4 M NaCl, and PBS/1% NP-40, and finally eluted with 4 M guanidine-Cl (pH 7.0). The enriched fusion protein was further purified on a G4000 column (TSK America Inc., North Bend, WA) and eluted at 0.5 ml/min with 0.1 M sodium phosphate buffer, pH 6.5, containing 0.1% SDS. The eluted protein was boiled and a portion of the antigen was crosslinked with 0.5% glutaraldehyde. These antigens were injected into a rabbit through multiple sites in 0.5-mg doses. Booster injections were given every 3 wk and antiserum was collected 4 d after each booster.

The antiserum obtained this way was extensively preadsorbed on induced λgt11 lysogens (without inserts) immobilized onto nitrocellulose membranes (3 ml antiserum/50,000 plaques per 10-cm filter) to remove nonspecific anti-*E. coli* and anti-β-galactosidase antibodies. Before this adsorption, nitrocellulose membranes were fixed with 10% acetic acid/15% isopropanol and subsequently blocked with 1% dry milk to saturate nonspecific sites. The preadsorbed antiserum was then further enriched by affinity chromatography by incubating this antiserum with blocked nitrocellulose membranes carrying replicas of induced λgt11 L3-5 lysogens prepared as before. Membranes were washed extensively with alternate washes of PBS, PBS/0.4

M Nacl, PBS/1% NP-40, and the membrane-bound antibodies were eluted with 0.1 M glycine, pH 2.2, and neutralized immediately with 0.1 vol of 1 M Tris-HCl, pH 8.0. After dialysis against PBS, these antibodies were used for immunofluorescence and immunoprecipitation studies.

Indirect immunofluorescence was carried out with cells sedimented onto slides in the cytocentrifuge that were then fixed with 1% paraformaldehyde for 10 min and permeabilized with acetone (−20°C for 3 min). After blocking with 1% goat serum in PBS, slides were stained with specific IgG (50 μg/ml) as the first antibody and visualized with FITC-conjugated goat anti-rabbit IgG $(Fab')_2$ (Boehringer Mannheim Biochemicals, Indianapolis, IN; 1:100 dilution). Slides were mounted with glycerol and observed under a Nikon fluorescence microscope.

For immunoprecipitation, subcellular fractions of cells were reacted with affinity-purified anti-L3-5 IgG (at 1 mg/10^8 cells) for 2 h, followed by incubation with protein A-agarose (Boehringer Mannheim Biochemicals) for an additional 2 h and sedimentation in the microfuge. The immunoprecipitate was resuspended in gel sample buffer containing 1% SDS and boiled for 5 min before its use in gel electrophoresis and autoradiography. Gel electrophoresis was performed on 10–20% gradient gels according to Laemmli (39). After being developed at constant current, the gels were soaked in EN^{3}HANCE (New England Nuclear) for 1 h, dried under vacuum, and subjected to autoradiography for 5–7 d.

[^{3}H]DFP Labeling of Proteins. [^{3}H]diisopropyl fluorophosphate ([^{3}H]DFP) labeling was performed essentially as described before (13). Briefly, to 100–200-μl protein samples, a reaction mixture of 1 M Tris-HCl buffer (pH 7.5), containing 1 mM [^{3}H]DFP (5.1 Cimmol^{-1}; New England Nuclear) was added to 10% final volume. After 60 min at 37°C, the reaction was terminated by addition of boiling buffer (2×) containing 2% SDS and 50 mM DTT, followed by additional boiling in Neville's buffer for 5 min. Samples were then applied directly to gel slabs and processed as before.

Results

Isolation of CTL-specific Serine Esterase cDNAs. We have recently isolated T lymphocyte subset-specific cDNAs from Th and CTL by means of a modified differential screening procedure (26). The specific expression of 21 potential CTL-specific cDNAs was further studied by Northern blot analysis of RNA from K46 B cells, unstimulated or TPA-stimulated EL-4 thymoma, unstimulated or Con A-stimulated L2 Th, unstimulated or Con A-stimulated L3 CTL, and rat LGL cells. Of these, eight inserts produced specific hybridization signals with L3 RNA while 13 inserts failed to show hybridization signal with any RNA used. We reasoned that the failure of the 13 inserts to hybridize with any RNA tested was probably due to the low expression of corresponding transcripts. Subsequently, nucleotide sequence analysis revealed that one clone among the eight inserts, L3G10 no. 6, was identical to H factor (22), while one clone among the latter 13 inserts, designated L3-1, represented a novel transcript with ~64% sequence homology to CCP1 (23).

L3G10 no. 6 is expressed in unstimulated and Con A-stimulated L3 cloned CTLs, but not in L2 Th, K46 B cells, EL-4 thymoma cells, or NK-like rat LGL (Fig. 1 *A*). L3G10 no. 6 is also expressed at a high level in another CTL line, CTLL-A11 (Fig. 1 *B*). On the other hand, L3-1 fails to detect mRNA in any of the RNA used in Fig. 1 *A* but detects 900 base mRNAs in CTLL-A11 cells (Fig. 1 *C*). L3-7 probe (Fig. 1 *D*) is an anonymous cDNA from L3 cDNA library and was used to demonstrate that each lane of the RNA blot used for Fig. 1 *B* and *C* contains similar amounts of RNA. For Fig. 1, *B–D*, the same filter was used successively in that order. These results appear to confirm the CTL specificity of L3-1 cDNA. Its level of expression is undetectable in L3 cells although the clone is originated from these same cells,

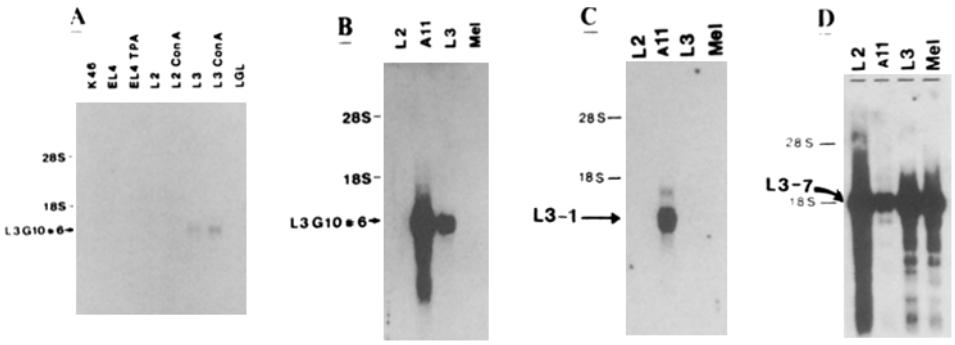

FIGURE 1. Specificity of expression of L3G10 no. 6 and MCSP-1. *A* shows that L3G10 no. 6 detects ~800 bases RNA in CTL clone L3. *B* shows that L3G10 no. 6 detects homologous transcript in both CTLL-A11 and L3; and *C* shows that MCSP-1 (L3-1 probe) detects the homologous transcript only in CTLL-A11. *D* shows that each lane of the RNA blot used for *B* and *C* contains similar amounts of RNA (L3-7 is an anonymous probe). (*B–D*) The same RNA blot was used to hybridize successively with L3G10 no. 6, MCSP-1 and L3-7 probes. Poly(A)$^+$ RNA was prepared from the following cells: K46, unstimulated and TPA-stimulated EL-4, rat LGL, CTLL-A11 and murine melanoma Cloudman S-91 cells (mel). Total RNA was prepared from unstimulated and Con A–stimulated L2 cells and unstimulated and Con A–stimulated L3 cells. 10 μg of total RNA or poly(A)$^+$ RNA was fractionated on a formaldehyde/agarose gel, transferred, and hybridized with ^{32}P-labeled L3G10 no. 6, L3-1, or L3-7. MCSP-1 consists of L3-1 and L3-5 cDNA inserts.

an observation that illustrates the usefulness of our differential screening procedure in detecting gene transcripts present in cells at low levels.

L3-1 contained a cDNA insert of 281 bp and was used to screen λgt11 L3 cDNA library to obtain a full-length version of L3-1. We obtained a cDNA clone L3-5 containing a 635-bp cDNA insert excluding the poly(A) tail. L3-1 and L3-5 shared 221 bp.

Fig. 2 *A* shows the nucleotide sequence and deduced amino acid sequence of the longest open reading frame derived from L3-1 and L3-5 cDNA inserts. The sequence was designated as a mouse CTL serine protease 1 (MCSP-1). Comparison between MCSP-1 and the CCP1/CTLA1 sequences revealed that MCSP-1 potentially did not contain ~30 amino acids equivalent in the 5′ coding sequence of the transcript. Therefore, a cDNA library prepared from CTLL-A11 mRNA was screened using L3-1 probe to obtain a full-length version of L3-1. In this screening, we isolated many cDNA clones that hybridized to L3-1 probe. Partial nucleotide sequencing of the 14 new cDNA inserts revealed that none of the 14 clones was identical to MCSP-1. The data suggest that MCSP-1 expression may also be very low in CTLL-A11 cells and that the hybridizing band seen in CTLL-A11 RNA (Fig. 1 *C*) is due to a cross-hybridization to L3-1-related sequences. Among the 14 clones, five cDNA clones whose insert sizes were >700 bases were analyzed further. The five clones represented two other related sequences, designated as MCSP-2 and MCSP-3.

Nucleotide and Deduced Amino Acid Sequences of the Three New Serine Esterase cDNAs. Fig. 2, *A–C* shows the nucleotide and deduced amino acid sequences of MCSP-1, MCSP-2, and MCSP-3. The homology of nucleotide sequence among the three cDNAs is ~64% and the nucleotide changes are dispersed along the cDNA sequence. Each open reading frame of MCSP-2 and MCSP-3 code for a potential mature polypeptide of 228 amino acids.

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A
  1    GGA GGC TTC TTG GTT CAA GAT GAT TTT GGC CTG ACG GCT GCT CAC TGC AGA AAC AGG TCA    60
  1    Gly Gly Phe Leu Val Gln Asp Asp Phe Gly Leu Thr Ala Ala His Cys Arg Asn Arg Ser    20
                                                                  Δ
 61    ATG ACA GTC ACA CTG GGG GCC CAC AAC ATC AAG GCT AAG GAG GAG ACA CAC CAG ATC ATC   120
 21    Met Thr Val Thr Leu Gly Ala His Asn Ile Lys Ala Lys Glu Glu Thr Gln Gln Ile Ile    40
121    CCT GTG GCA AAA GCC ATT CCC CAT CCA GCT TTT AAT AGA AAG CAT GGC ACC AAT GAC ATT   180
 41    Pro Val Ala Lys Ala Ile Pro His Pro Ala Phe Asn Arg Lys His Gly Thr Asn Asp Ile    60
                                                                              Δ
181    ATG TTA TTA AAA CTG GAG AGT AAG GCC AAG AGA ACT AAA GCT GTG AGG CCC CTC AAG TTG   240
 61    Met Leu Leu Lys Leu Glu Ser Lys Ala Lys Arg Thr Lys Ala Val Arg Pro Leu Lys Leu    80
241    CCC AGA CCC AAT GCC AGG GTG AAG CCA GGG GAT GTG TGC AGT GTG GCT GGC TGG GGG AAA   300
 81    Pro Arg Pro Asn Ala Arg Val Lys Pro Gly Asp Val Cys Ser Val Ala Gly Trp Gly Lys   100
301    ACA TCC ATC AAT GCC ACT AAA GCA TCT GCC CGC CTG CGA GAG GCT CAA CTG ATC ATC CAG   360
101    Thr Ser Ile Asn Ala Thr Lys Ala Ser Ala Arg Leu Arg Glu Ala Gln Leu Ile Ile Gln   120
361    GAG GAC GAG GAA TGC AAA AAA CTC TGG TAC ACC TAT TCC AAG ACC ACG CAG ATC TGT GCT   420
121    Glu Asp Glu Glu Cys Lys Lys Leu Trp Tyr Thr Tyr Ser Lys Thr Thr Gln Ile Cys Ala   140
421    GGA GAC CCA AAA AAA GTA CAG GCT CCT TAC GAG GGT GAA TCT GGG GGT ACC CTC GTG TGT   480
141    Gly Asp Pro Lys Lys Val Gln Ala Pro Tyr Glu Gly Glu Ser Gly Gly Thr Leu Val Cys   160
                          *                              Δ
481    GAC AAC CTA GCT TAT GGA GTT GTA TCC TAT GGA ATA AAC AGG ACA ATC ACT CCA GGA GTC   540
161    Asp Asn Leu Ala Tyr Gly Val Val Ser Tyr Gly Ile Asn Arg Thr Ile Thr Pro Gly Val   180
541    TTC ACT AAG GTT GTG CAC TTC CTG CCG TGG ATA AGC ACA AAC ATG AAG CTG CTC TAA CAG   600
181    Phe Thr Lys Val Val His Phe Leu Pro Trp Ile Ser Thr Asn Met Lys Leu Leu
601    GAG TTA AAC CAC CCG TGC CTG ACC AGC CTG TCC GAC CTC AGG CAA GAA CCA TGT GGA GTG   660
661    AGC AGC AAA GAA TGA AAA TTC ATA ATA AAT AAC CTC CAG AGT GCA TAA AAA AAA AAA AAA   720

B
                                                    1  CTT GGA GCT GGA GCA GAG GAG    21
                                                   -7  Leu Gly Ala Gly Ala Glu Glu    -1
                                                                                 Δ
                                                                                 ↑
 22    ATC ATC GGC GGC CAT GTG GTG AAG CCA CAC TCC CGC CCC TAC ATG GCG TTT GTT AAG TCT    81
  1    Ile Ile Gly Gly His Val Val Lys Pro His Ser Arg Pro Tyr Met Ala Phe Val Lys Ser    20
 82    GTG GAT ATT GAA GGT AAT AGG AGA TAC TGT GGA GGC TTC TTG GTT CAA GAT GAC TTT GTG   141
 21    Val Asp Ile Glu Gly Asn Arg Arg Tyr Cys Gly Gly Phe Leu Val Gln Asp Asp Phe Val    40
142    CTG ACT GCT GCT CAC TGC AGG AAC AGG ACA ATG ACA GTC ACA CTG GGG GCC CAC AAC ATC   201
 41    Leu Thr Ala Ala His Cys Arg Asn Arg Thr Met Thr Val Thr Leu Gly Ala His Asn Ile    60
                     Δ
202    AAG GCT AAG GAG GAG ACA CAG CAG ATC ATC CCT GTG GCA AAA GCC ATT CCC CAT CCA GAT   261
 61    Lys Ala Lys Glu Glu Thr Gln Gln Ile Ile Pro Val Ala Lys Ala Ile Pro His Pro Asp    80
262    TAT AAT GCC ACT GCC TTC TTC AGT GAC ATC ATG CTG TTA AAG CTG GAG AGT AAG GCC AAG   321
 81    Tyr Asn Ala Thr Ala Phe Phe Ser Asp Ile Met Leu Leu Lys Leu Glu Ser Lys Ala Lys   100
                                    Δ
322    AGA ACT AAA GCT GTG AGA CCC CTC AAG TTG CCC AAG CCC AAT GCC CGG GTG AAG CCA GGG   381
101    Arg Thr Lys Ala Val Arg Pro Leu Lys Leu Pro Lys Pro Asn Ala Arg Val Lys Pro Gly   120
382    GAT GTG TGC AGT GTG GCT GGC TGG GGG TCA AGG TCC ATC AAT GAC ACT AAA GCA TCT GCC   441
121    Asp Val Cys Ser Val Ala Gly Trp Gly Ser Arg Ser Ile Asn Asp Thr Lys Ala Ser Ala   140
442    CGC CTG CGA GAG GCT CAA CTG GTC ATC CAG GAG GAT GAG GAA TGC AAA AAA CGT TTC CGA   501
141    Arg Leu Arg Glu Ala Gln Leu Val Ile Gln Glu Asp Glu Glu Cys Lys Lys Arg Phe Arg   160
502    CAC TAC ACT GAG ACC ACA GAG ATT TGT GCT GGA GAC TTG AAG AAA ATA AAG ACT CCT TTC   561
161    His Tyr Thr Glu Thr Thr Glu Ile Cys Ala Gly Asp Leu Lys Lys Ile Lys Thr Pro Phe   180
                                                                              *
562    AAG GGT GAC TCT GGG GGA CCC CTC GTG TGT GAC AAC AAA GCT TAT GGA CTT TTA GCC TAT   621
181    Lys Gly Asp Ser Gly Gly Pro Leu Val Cys Asp Asn Lys Ala Tyr Gly Leu Leu Ala Tyr   200
                  Δ
622    GCA AAA AAC AGG ACA ATC TCT TCA GGA GTC TTC ACT AAG ATT GTG CAC TTC CTG CCG TGG   681
201    Ala Lys Asn Arg Thr Ile Ser Ser Gly Val Phe Thr Lys Ile Val His Phe Leu Pro Trp   220
682    ATA AGC AGG AAC ATG AAG CTG CTC TAA CAG GTG TTA AAC CAC CCG TGC CTG ACC AGC CTG   741
221    Ile Ser Arg Asn Met Lys Leu Leu ---
742    TCC GAC CTC AGG CAA GAA CCA CGT GGA GTG GGC AGC AAA GAA TGA AAA TTC ACA A

C
-160                                                GGCTTCTGCT CCAGTGCCAC CCTGATGCAG GCCTCCTGAG AACTTTTCCG ATGCTTCCCT
-100  CTCTCCTTGG GCCACACCCG CTCTCTACAG ACCTCCTTCC TCCCCTTCCA AGCCCTTTGT CTACTCTGCC CTCCTCCATC CTGAGCAGTC CTTTATTCAG

  1  ATG CCA CCA ATC CTG ATT CTC CTG ACC CTT CTT CTG CCT CTC AGA GCT GGA GCA GAG GAG    60
-20  Met Pro Pro Ile Leu Ile Leu Leu Thr Leu Leu Leu Pro Leu Arg Ala Gly Ala Glu Glu    -1
 61  ATC ATC GGG GGC CAT GAG GTC AAG CCC CAC TCC CGC CCT TAC ATG GCA CGT GTG AGG TTT   120
  1  Ile Ile Gly Gly His Glu Val Lys Pro His Ser Arg Pro Tyr Met Ala Arg Val Arg Phe    20
121  GTG AAA GAT AAT GGA AAA AGA CAT TCC TGT GGA GGC TTC CTG GTT CAA GAC TAC TTT GTG   180
 21  Val Lys Asp Asn Gly Lys Arg His Ser Cys Gly Gly Phe Leu Val Gln Asp Tyr Phe Val    40
181  CTG ACG GCT GCT CAC TGC ACT GGA AGC TCA ATG AGA GTC ATA CTG GGG GCC CAC AAC ATC   240
 41  Leu Thr Ala Ala His Cys Thr Gly Ser Ser Met Arg Val Ile Leu Gly Ala His Asn Ile    60
                  Δ
241  AGG GCT AAG GAA GAG ACA CAG CAG ATC ATC CCT GTG GCA AAA GCC ATT CCC CAC CCA GCT   300
 61  Arg Ala Lys Glu Glu Thr Gln Gln Ile Ile Pro Val Ala Lys Ala Ile Pro His Pro Ala    80
301  TAT GAT GAT AAG GAC AAC ACC AGT GAC ATC ATG CTA TTA AAG CTG GAG AGT AAG GCC AAG   360
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FIGURE 2. Legend appears on opposing page.

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 81   Tyr Asp Asp Lys Asp Asn Thr Ser Asp Ile Met Leu Leu Lys Leu Glu Ser Lys Ala Lys    100
                                      Δ
361   AGA ACT AAA GCT GTG AGG CCC CTC AAG TTG CCC AGA CCC AAT GCC CGG GTG AAG CCA GGG    420
101   Arg Thr Lys Ala Val Arg Pro Leu Lys Leu Pro Arg Pro Asn Ala Arg Val Lys Pro Gly    120
421   CAT GTT TGC AGT GTG GCT GGC TGG GGG AGA ACA TCC ATC AAT GCA ACA CAA AGA TCT TCC    480
121   His Val Cys Ser Val Ala Gly Trp Gly Arg Thr Ser Ile Asn Ala Thr Gln Arg Ser Ser    140
481   TGC CTA CGA GAG GCT CAA CTG ATC ATC CAG AAG GAT AAG GAA TGC AAA AAA TAC TTC TAT    540
141   Cys Leu Arg Glu Ala Gln Leu Ile Ile Gln Lys Asp Lys Glu Cys Lys Lys Tyr Phe Tyr    160
541   AAG TAT TTC AAG ACC ATG CAG ATT TGT GCT GGA GAC CCA AAG AAA ATA CAG TCT ACT TAC    600
161   Lys Tyr Phe Lys Thr Met Gln Ile Cys Ala Gly Asp Pro Lys Lys Ile Gln Ser Thr Tyr    180
                                                                          *
601   AGT GGT GAC TCC GGG GGA CCC CTC GTG TGT AAC AAC AAA GCT TAT GGA GTT TTA ACC TAT    660
181   Ser Gly Asp Ser Gly Gly Pro Leu Val Cys Asn Asn Lys Ala Tyr Gly Val Leu Thr Tyr    200
                 Δ
661   GGG CTA AAC AGG ACA ATC GGT CCA GGA GTC TTC ACT AAG GTT GTG CAC TAC CTG CCG TGG    720
201   Gly Leu Asn Arg Thr Ile Gly Pro Gly Val Phe Thr Lys Val Val His Tyr Leu Pro Trp    220
721   ATA AGC AGG AAC ATG AAG CTG CTC TAA CAG GAG TTA AAC CAC CCG TGC CTG ACC AGC CTG    780
221   Ile Ser Arg Asn Met Lys Leu Leu ---
781   TCC GAC CTC AGG CAA GAA CCA TGT GGA GTG AGC AGC AAA GAA TGA AAA TTC ATA ATA AAT    840
841   AAC CTC CAG AGT GCA TAG AGC TGA AAA
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FIGURE 2. Nucleotide sequence and the deduced amino acid sequence of MCSP-1 (*A*),MCSP-2 (*B*), and MCSP-3 (*C*). The nucleotide sequences of message strands are numbered in 5′ to 3′ direction. The amino acids forming a serine esterase active site (Δ) are His^{45}, Asp^{89}, and Ser^{184}. The potential asparagine-linked glycosylation sites are underlined. The putative active site pocket (*) is at residue Ala in MCSP-1, Thr in MCSP-2, and Ser in MCSP-3. The AATAAA polyadenylation consensus sequence is boxed in the 3′ noncoding region in MCSP-1 and MCSP-3 sequences. Stop codon (---). An arrow indicates a putative cleavage site for active enzyme.

The molecular weights corresponding to the mature protein backbones of MCSP-2 and MCSP-3 are 25,477 and 25,360, respectively. There are three potential glycosylation signals in MCSP-1 and MCSP-3, and four in MCSP-2 as underlined in Fig. 2, *A–C*. The codon specifying COOH-terminal leucine is followed by the translation termination codon TAA in all three sequences. MCSP-1 and MCSP-3 contain a potential polyadenylation signal of AATAAA (boxed nucleotides).

Fig. 3 *A* shows the optimal alignment of the three peptide sequences. The NH_2-terminal amino acids (*underlined residues*) of MCSP-2 and MCSP-3 proteins are identical to those reported for two granule serine esterases which, according to the nomenclature proposed by Masson and Tschopp (14), would correspond to granzymes E and F, respectively (15). Three residues (His^{45}, Asp^{89}, and Ser^{184}), which are known to form a serine esterase active site (40), are found in the same position in the three proteins. The active site pocket residue positioned six residues before the active-site Ser^{184} (41, 42) is alanine in MCSP-1, threonine in MCSP-2, and serine in MCSP-3 (Fig. 3 *A*, *asterisks*).Thus, MCSP-2 and MCSP-3 may have chymotrypsin-like specificity. The predicted amino acids show 76% homology between MCSP-1 and MCSP-2, 80% homology between MCSP-1 and MCSP-3, and 73% homology between MCSP-2 and MCSP-3.

Since the predicted amino acid sequence for MCSP-1 is substantially different from other reported T cell serine proteases, the amino acid sequence of MCSP-1 was compared with other serine protease-like proteins whose active site pocket residue is alanine. Fig. 3 *B* shows a comparison of predicted amino acids of MCSP-1 with those of CCP1 (23), granzyme C (25), RMCPII (43, 44) and cathepsin G (45). Approximately 33% of the amino acids are identical in all five proteins (Fig.3 *B*, *boxed amino acids*). The amino acid identity was 56% between MCSP-1 and CCP1, 58% between MCSP-1 and granzyme C, 42% between MCSP-1 and RMCPII, and 46% between MCSP-1 and cathepsin G. The serine protease triads are conserved

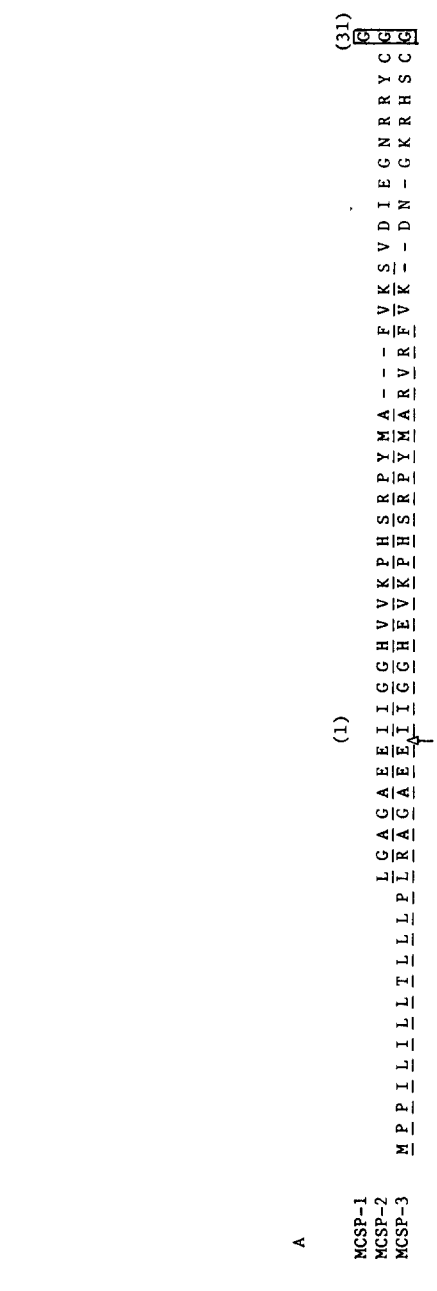

FIGURE 3. Legend appears on opposing page.

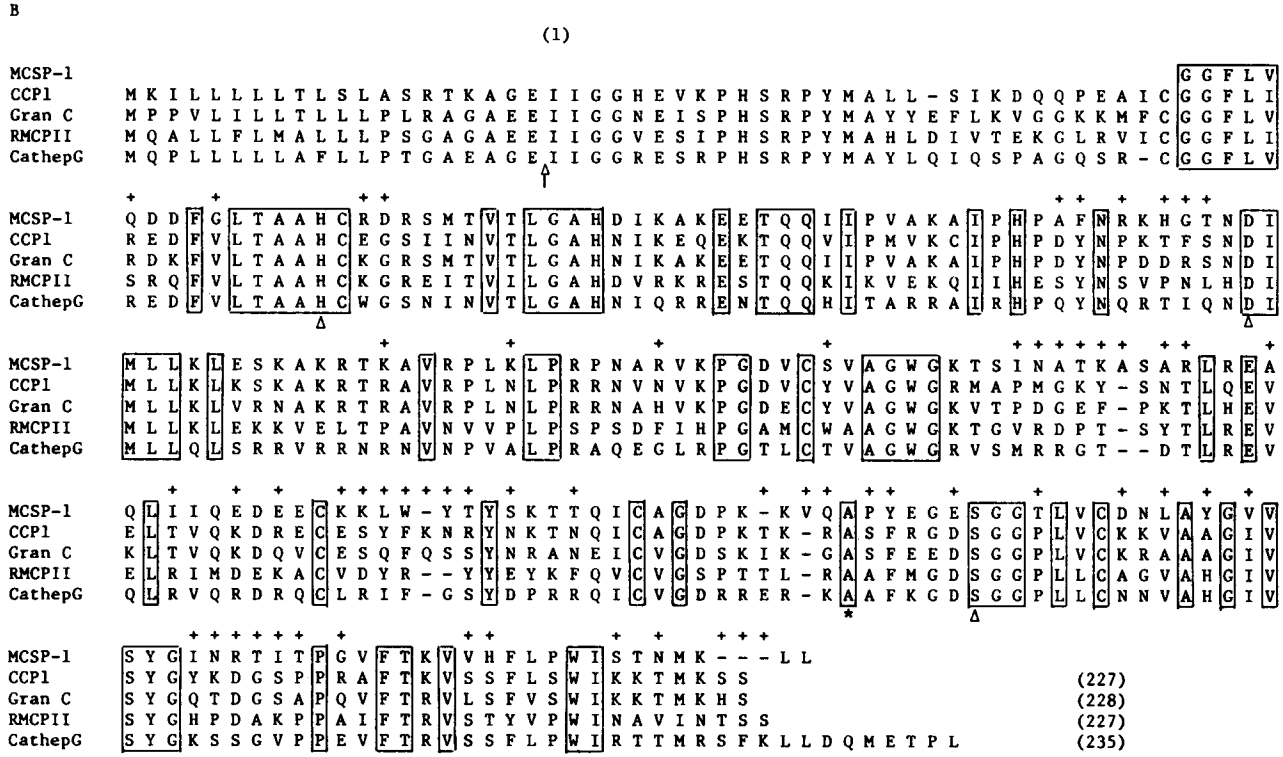

FIGURE 3. Alignment of predicted amino acid sequences of T cell serine proteases. Comparisons were made among MCSP-1, MCSP-2, and MCSP-3 deduced amino acid sequences (*A*), andMCSP-1 amino acids were aligned with four other predicted serine proteases whose active site pocket residue is alanine (*B*). An arrow indicates a putative cleavage site to generate an active enzyme. (Δ) The "charge-relay" system of the active site of serine proteases; (*) the potential amino acid residue that participates in the primary substrate binding site in serine proteases. The numbering begins at the NH_2 terminus of the predicted active enzyme. Gaps were introduced to optimize the alignment. Amino acids that are identical among the sequences are boxed. Numbers in parentheses indicate the positions of amino acids. (*A*) The underlined NH_2-terminal amino acids of MCSP-2 and MCSP-3 are identical to those of granzymes E and F, respectively. (*B*) Positions that the amino acids of MCSP-1 are different from those of other four proteins (+).

in similar space in all five proteins (Fig. 3 *B*, indicated by Δ). However, ~30% of amino acids of MCSP-1 (60 positions indicated by + in Fig. 3 *B*) are different from those of the other four proteins. These data may indicate that MCSP-1 is a new member of T cell serine protease family.

Southern Blot Analysis. Southern blot analysis of mouse (C57BL/6) genomic DNA was performed with MCSP-1, MCSP-2, andMCSP-3 clones (Fig. 4, *A–C*, respectively). All three cDNAs detect the same strongly hybridizing bands but detect weakly hybridizing bands differentially. For example, when the mouse DNA is digested with Bam H1, all three cDNAs detect three strong bands whose sizes are 10.9, 7.4, and 2.5 kb. In addition, there are three weakly hybridizing bands whose sizes are 6.0, 5.0, and 2.6 kb. Of these, three weak bands are detected by MCSP-3 but only two bands each hybridize to MCSP-1 and MCSP-2 with different intensity. A similar phenomenon is observed in Eco RI and Hind III–digested genomic DNA (Fig. 4). These hybridization data and sequence information suggest that there is a family of genetic loci that contains separate genes representing these three cDNAs. Their exon and intron composition must be similar.

Antibodies Derived Against Fusion Protein Carrying MCSP-1 React with Granule Proteins. Direct sequence analysis with a λgt11 forward primer revealed that L3-5 cDNA was in frame with the *lacZ* gene of the λgt11 vector. Thus, the L3-5 clone was used to prepare antibodies against the fusion protein.

Lysogens harboring the L3-5 clone were induced with IPTG. The fusion protein was purified by immunoaffinity chromatography using agarose-bound β-galactosidase-specific mAbs as the immunoadsorbent. A rabbit antiserum raised against this fusion protein was further enriched for MCSP-1-specific antibodies through a differential adsorption method detailed in Materials and Methods. Affinity-purified

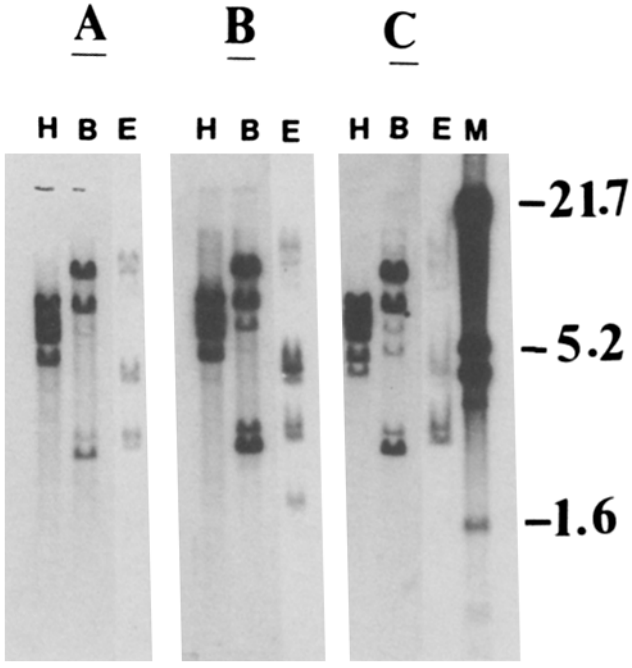

FIGURE 4. Southern blot analysis of mouse DNA. Genomic DNA from C57BL/6 liver was digested with Hind III (*H*), Bam HI (*B*), or Eco RI (*E*), electrophoresed on a 0.8% agarose gel, transferred to Gene Screen Plus, and hybridized to MCSP-1 (*A*), MCSP-2 (*B*), and MCSP-3 (*C*). (*M*) Molecular size marker in kilobase pairs.

antibodies were then used to stain various cell lines by indirect immunofluorescence. CTLL-A11, CTLL-1, and CTLL-R8, but not EL-4 thymoma or YAC-1 leukemia cells, were stained with these antibodies (not shown). Granulated staining patterns were always observed, suggesting the predominant granular localization of the MCSP-1 protease (Fig. 5). In parallel experiments no reactivity was given by the preimmune serum control. To assess the distribution of this antigen in vivo, anti-MCSP-1 antibodies were next used to stain bulk cell populations from murine spleen, liver, and peripheral blood. Staining was persistently negative for all the cell populations examined. However, lymphocyte populations that had been stimulated with IL-2 for 3–7 d displayed weakly positive stain in their granules. Positive staining was associated mainly with a subpopulation of LGL that had been enriched by their selective adherence to plastic dishes (not shown; see Materials and Methods). Nonadherent cells did not show any detectable reactivity. In parallel experiments murine primary CTL (3–5 d old), obtained from bulk spleen lymphocyte populations by MLRs, also displayed faint reactivity to the antibody as determined by immunofluorescence. These results suggest that MCSP-1-related antigens are induced to measurable levels in both alloimmune-stimulated CTL and IL-2-driven LGL/NK cells. Since L3-1 (a murine MCSP-1-related transcript) did not hybridize with the mRNA of rat tumor LGL (Fig. 1 *A*), these results would argue that either there are some significant differences between the mouse and the rat transcripts that would restrict crosshybridiza-

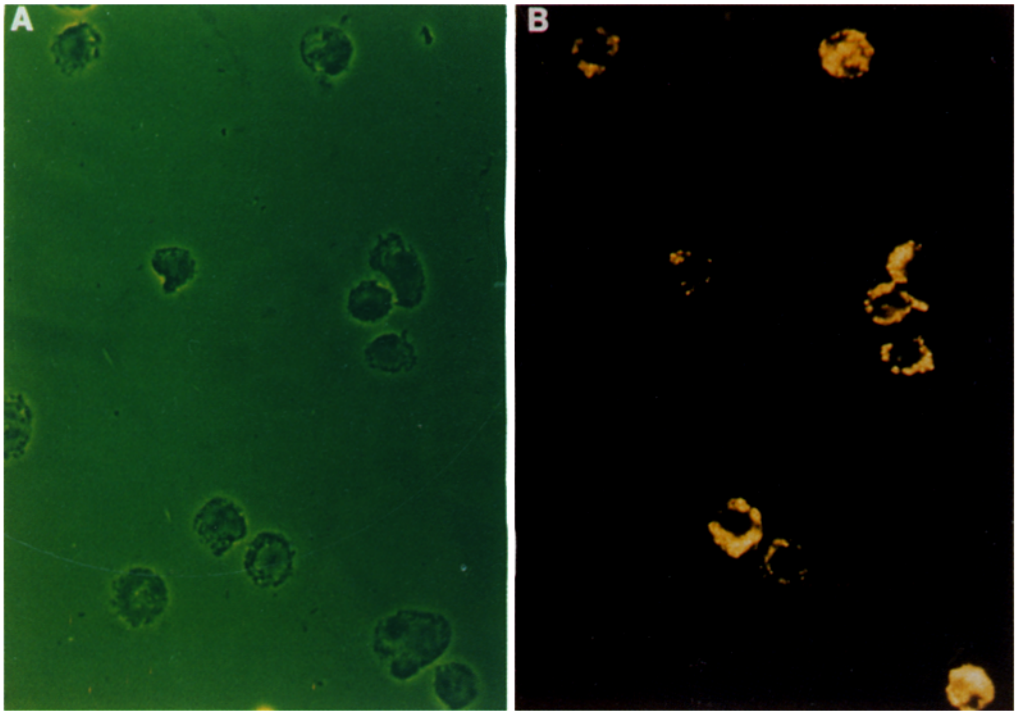

FIGURE 5. Indirect immunofluorescence using anti-L3-5 fusion protein antibodies. CTLL-A11 cells, sedimented in the cytocentrifuge, were stained with affinity-purified antibodies prepared against L3-5 β-galactosidase fusion protein. (*A*) Phase-contrast; (*B*) same field, fluorescence; ×600. The preimmune control serum produced undetectable reactivity (not shown).

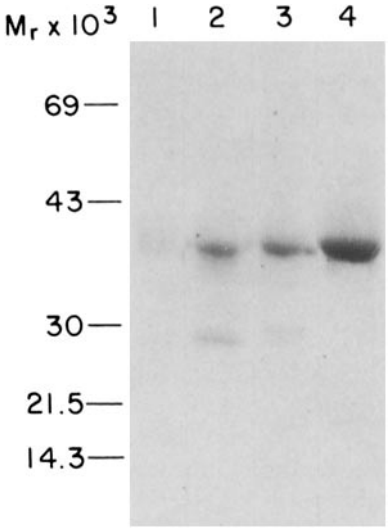

FIGURE 6. Immunoprecipitation of CTL proteins with anti-L3-5 fusion protein antibodies and [^{3}H]DFP labeling. (Lanes *1-3*) Immunoprecipitation of [^{35}S]methionine-labeled CTLL-A11 (lanes *1* and *2*) and CTLL-R8 cells (lane *3*). Lanes *2* and *3* contain proteins from granule-enriched pellet that have been reacted with affinity-purified anti-L3-5 fusion protein IgG (see Materials and Methods). The precipitation profile corresponds to material derived from 10^8 cells. (Lane *1*) Precipitation was carried out with granule-depleted cytosol under exact conditions as described above; material also corresponds to 10^8 cell equivalents. The autoradiogram was exposed for 5 d. (Lane *4*) [^{3}H]DFP labeling of immunoprecipitated proteins. Immunoprecipitation of CTLL-A11 granule-enriched proteins was carried out exactly as in lane *2* except that cells were not labeled. The immunoprecipitate was reacted with [^{3}H]DFP as described in Materials and Methods and loaded onto the gel. Note that only the 35-40-kD protein band labels strongly with [^{3}H]DFP. Autoradiography was performed for 3 d.

tion or that the rat tumor LGL (RNK cells) used here are not truly representative of bulk LGL populations obtained from the spleen.

Antibodies were next reacted with cell proteins in immunoblots (Fig. 6). CTLL-A11 were labeled with [^{35}S]methionine, cavitated under N_2, and the nucleus-free lysate was centrifuged at 13,000 *g* for 20 min. The pellet obtained under these conditions contained granules and other cellular organelles as judged by a 10–20-fold enrichment of trypsin-like serine esterase and hemolytic (pore-forming) activities. Immunoprecipitation of high-salt buffer-extracted pellet proteins with anti-MCSP-1 antibodies yielded a strong band with a molecular mass of 35–40 kD and a weaker band of 29 kD (Fig. 6, lane *2*). Some reactivity (10–30% of the total cell label) was also found in the granule supernatant (Fig. 6, lane *1*). A similar precipitation profile was also found associated with another CTL line, CTLL-R8 (Fig. 6, lane *3*). Preimmune control serum did not precipitate any specific band (not shown). The 35–40-kD band labeled strongly with [^{3}H]DFP (Fig. 6, lane *4*), a specific affinity label for serine proteases, whereas the 29-kD band labeled weakly or not at all. These results are consistent with the data presented recently by Masson and Tschopp (14), who also described variable DFP reactivities for the various granule proteases they have identified. Since MCSP-1 is known to be structurally related to the two other granule serine proteases, antibodies raised against MCSP-1 fusion protein may have also crossreacted with other granule species.

Discussion

To isolate mRNA species, including rare transcripts specific to a cell type, we developed a protocol for differential screening without prior selection and applied the approach to the analysis of cDNA libraries from Con A-stimulated helper (L2) and CTL (L3) cells. In the above analysis, we could not detect transcripts representing 30–40% of cDNA inserts from L2 and L3 cells. We speculated that those inserts could still be subset specific, but that the L2 and L3 RNA loads used in the RNA blot analysis were not high enough to detect a basal level of these RNA species. In fact, one of the cDNA inserts, L3-1, corresponded to a CTL-specific serine protease

that was originated from L3 cells but whose RNA expression in these cells was too low to detect. On the other hand, the expression of the MCSP-1-related cDNAs, MCSP-2 and MCSP-3, was very high in another CTL clone, CTLL-A11. Since we could not clone MCSP-1 cDNA from the CTLL-A11 cDNA library, this sequence must not be expressed at high level in the CTLL-A11 either. A 23-mer oligo nucleotide matching the 5′ end of L3-1 sequence was used to prime cDNA synthesis on poly $(A)^+$ mRNA from CTLL-A11 cells (data not shown). The primer extension products were undetectable in many repeated experiments, which was another indication that L3-1 mRNA level was low in CTLL-A11 cells. Notably, CTLL-A11 expressed H factor/CTLA 3, MCSP-2, and MCSP-3 at high levels, whereas L3 cells expressed H factor/CTLA 3 only at high level, while MCSP-1, MCSP-2, and MCSP-3 transcripts were expressed at undetectable levels, suggesting marked heterogeneity in the expression of serine esterase messages even among different CTL clones.

Based upon the identity of NH_2-terminal amino acids of MCSP-2 and MCSP-3 with the reported partial sequence of the so-called granzymes E and F (15), we assigned MCSP-2 as granzyme E and MCSP-3 as granzyme F. MCSP-1 differs from the published sequences of granzymes A–F (15). Masson and Tschopp (14) observed that granzymes D–F are closely related in that they all react with antibodies to granzyme D and are similar in their degree of glycosylation and the position of at least one tryptophan residue in their polypeptide chains. As shown in Fig. 3 *A*, there are indeed two tryptophans that appear at the same positions in all three deduced protein sequences presented here. Antibodies raised against L3-5 β-galactosidase fusion proteins react strongly with a 35–40-kD protein, which corresponds to the presumed molecular mass of granzyme D. It should be noted, however, that the number of potential *N*-glycosylation sites are three for MCSP-1 instead of five for granzyme D and that the active site pocket residue is alanine for MCSP-1 instead of threonine for granzyme D. Therefore, MCSP-1 protein may be a seventh member of murine T cell serine protease family.

With the completion of this work, six closely related serine esterase transcripts (granzymes A, B, C, E, F, and MCSP-1) will have been cloned and sequenced, fully supporting the notion that a family of serine esterases is found in lymphocyte granules. However, the function of these enzymes still remains to be determined (46). The three cDNAs described here were expressed only in CTL in our limited survey (Fig. 1, *A* and *B*). These transcripts may not be unique to CTL since at least one of them (MCSP-1 or a closely related product) was also expressed weakly by LGL/NK cells established in primary cultures. Studies with a large number of cell lines and more specific probes will be required to address vigorously the cell and tissue distribution of these cloned transcripts.

Serine esterases may be used as sensitive markers for lymphocytes that have been committed to become killer cells (10, 46). It is intriguing that many of the transcripts that have been cloned so far from subtractive CTL-specific cDNA libraries should turn out to code for serine proteases. T cell subsets may acquire such transcripts only upon activation via IL-2 or allo-immune stimulation. Serine esterase-specific probes could in principle be used to study the role of killer lymphocytes in health and in disease, particularly in the assessment of killer lymphocyte distribution in tissues afflicted with auto-immune disorders. The development of more specific nucleic acid probes and peptide-specific antisera for the various serine esterases should facilitate further studies on their function and expression.

Summary

Three new cDNA clones (designated MCSP-1, MCSP-2, and MCSP-3) encoding mouse serine proteases were isolated from cloned cytolytic T lymphocytes (CTL) by a modified differential screening procedure. The putative mature proteins of MCSP-2 and MCSP-3 are each composed of 228 amino acids with molecular weights of 25,477 and 25,360, respectively. NH_2-terminal amino acids of MCSP-2- and MCSP-3-predicted proteins were identical to those reported for granzyme E and F, respectively. The third species, MCSP-1, was closely related to the two other cDNA species but ~30 amino acids equivalents of the NH_2-terminal portion of the cDNA were not cloned. The amino acids forming the active sites of serine proteases were well conserved among the three predicted proteins. The active site pocket residue positioned six residues before the active-site Ser^{184} is alanine in MCSP-1, threonine in MCSP-2, and serine in MCSP-3, indicating that both MCSP-2 andMCSP-3 may have chymotrypsin-like specificity. There are three potential asparagine-linked glycosylation sites in MCSP-1 and MCSP-3, and four in MCSP-2-deduced amino acid sequences. Amino acid comparison of MCSP-1 with four other reported serine proteases whose active site pocket residue is alanine revealed that MCSP-1 was substantially different from the other molecules, indicating that MCSP-1 may be a new member of mouse T cell serine protease family. Antibodies made against a MCSP-1 *lacZ* gene fusion protein stain granules of CTL and react on immunoblots with two distinct granule protein bands of 29 and 35–40 kD. Only the 35-kD species labels with [^{3}H]DFP. Since a protease cascade may play a key role in cytolytic lymphocyte activation, our isolation of cDNAs representative of unique serine esterases should help to investigate such a cascade process.

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Note added in proof: Jenne et al. (47) reported cDNA sequence for granzyme D, E, and F. Amino acid comparison revealed that MCSP-2 was identical to granzyme E except for four amino acid changes at positions 85 (Ala–Ile), 87 (Phe–Tyr), 112 (Lys–Arg), and 130 (Ser–Pro), and that MCSP-3 was identical to granzyme F at all positions. MCSP-1 was different from granzyme D sequence.

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