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Immunology

Casein expression in cytotoxic T lymphocytes

MICHAEL J. GRUSBY*, STEVE C. MITCHELL*, NASRIN NABAVI*†, AND LAURIE H. GLIMCHER‡

*Department of Cancer Biology, Harvard School of Public Health, Boston, MA 02115; and ‡Department of Medicine, Harvard Medical School, Boston, MA 02115

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ABSTRACT **A cDNA that expresses a mRNA restricted to cytotoxic T lymphocytes (CTL) and mammary tissue has been isolated and characterized. The deduced amino acid sequence from this cDNA shows extensive homology with the previously reported amino acid sequence for rat α-casein. Indeed, the presence of a six-residue-repeated motif that is specific for rodent α-caseins strongly supports the identification of this cDNA as mouse α-casein. Northern (RNA) blot analysis of many hematopoietic cell types revealed that this gene is restricted to CTL, being expressed in four of six CTL lines examined. Furthermore, CTL that express this gene were also found to express other members of the casein gene family, such as β- and κ-casein. These results suggest that caseins may be important in CTL function, and their potential role in CTL-mediated lysis is discussed.**

While the mechanism by which cytotoxic T lymphocytes (CTL) bring about the lysis of target cells is not completely understood, several molecules thought to be involved in CTL effector function have been identified. There is now considerable evidence suggesting that perforin is an important mediator of cytolysis (1–3). Perforins, together with proteins possessing serine esterase activity (4–11), have been localized to dense cytoplasmic granules within both CTL (12) and natural killer cells (13), and the granule-exocytosis model for cytolysis suggests that these mediators are released by CTL upon antigenic stimulation. Although other molecules, such as cytotoxin (14) and a lipase recently described by our laboratory (15), have also been implicated in cytolysis, whether all components of the cytolytic apparatus have been identified is unclear. In this report, we characterize a cDNA§ that is expressed only in CTL. This cDNA probably encodes mouse α-casein, based upon similarity to rat α-casein and its tissue distribution. Given the ability of caseins to form calcium-dependent micelles (16) and the previous demonstration that casein is a substrate for serine esterases (17, 18), we propose a mechanism by which these two molecules, along with perforin, contribute to CTL-mediated cytolysis.

MATERIALS AND METHODS

Preparation of CT.4R(IL-4) cDNA Library. Poly(A)$^+$ RNA was prepared from CT.4R cells grown in recombinant interleukin 4 (IL-4) (Immunex, Seattle, WA) at 500 units/ml by oligo(dT)-cellulose (New England Biolabs) chromatography of total cellular RNA isolated by the guanidine thiocyanate method (19). First-strand cDNA synthesis was performed by using 4 μg of poly(A)$^+$ RNA, oligo (dT) primer (Promega), and cloned Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). Second-strand cDNA synthesis was accomplished by using DNA polymerase I (New England Biolabs) and RNase H (Promega). *Bst*XI linkers having the sequences 5′-CTCTAAAG-3′ and 5′-CTTTAGAGCACA-3′ were synthesized, phosphorylated, and ligated to the double-stranded cDNA with T4 DNA ligase (New England Biolabs). Free linkers and cDNA fragments of <800 base pairs (bp) were removed by Sepharose CL-4B (Pharmacia) chromatography, and the remaining cDNA was ligated to *Bst*XI-digested CDM8 plasmid vector (20). The ligated DNA was transformed into *Escherichia coli* MC1061/P3, and ≈1 × 10^6 recombinants were obtained.

Differential Hybridization Screening of CT.4R(IL-4) cDNA Library. Differential colony hybridization was done essentially as described (21). Approximately 10,000 individual colonies were each inoculated into 100 μl of L broth containing tetracycline at 7.5 μg/ml and ampicillin at 12.5 μg/ml in 96-well round-bottom microtiter plates. After an overnight incubation at 37°C, an equal volume of sterile glycerol was added to each well. Three replica filters (GeneScreen, New England Nuclear) were prepared from each 96-well plate by using a replica tool, and after overnight growth at 37°C, the filters were processed as described (21). Prehybridization was performed for 1 hr at 42°C in 48% (vol/vol) formamide/5× SSC (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7)/10 mM Tris·HCl, pH 7.6/1× Denhardt's solution (1× Denhardt's solution is 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/1% SDS/10% (wt/vol) dextran sulfate containing salmon sperm DNA at 100 μg/ml. Hybridization was performed for 48 hr and was initiated by adding ^{32}P-labeled cDNA probe (1 × 10^6 cpm/ml) synthesized from poly(A)$^+$ RNA obtained from CT.EV cells grown in recombinant interleukin 2 (IL-2) (20 units/ml), CT.4R cells grown in recombinant IL-4 (500 units/ml), or BW5147 thymoma cells. Filters were washed in 2× SSC/0.1% SDS for 40 min at room temperature and in 0.2× SSC/0.1% SDS for 40 min at 63°C. After 24-hr exposure at room temperature, those colonies that preferentially hybridized with probes derived from CT.EV(IL-2) or CT.4R(IL-4) cells were scored and rescreened under the conditions described above.

DNA Sequence and Computer Analysis. The 1.1-kilobase (kb) cDNA insert from clone 1F3 was subcloned into the *Xho* I site of the phagemid pBluescript (Stratagene) in both orientations. The most 5′ 237 nucleotides of the full-length sequence of this cDNA was obtained by polymerase chain reaction (PCR) amplification of cDNA, prepared from total cellular CT.4R RNA, by using oligonucleotides derived from the 5′-untranslated region of the rat α-casein cDNA (5′-TCTTGAATTCAAGATCTTAGCAACCA-3′) and from the internal region of clone 1F3 (5′-GGAGAAGTTGGTTG-TACT-3′). The nucleic acid sequences of both strands of clone 1F3 and the PCR product were determined by the dideoxynucleotide chain-termination method (22) with synthetic oligonucleotide primers. Sequence analysis was per-

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Abbreviations: CTL, cytotoxic T lymphocyte(s); PCR, polymerase chain reaction; IL-2 and -4, interleukin 2 and interleukin 4, respectively.

†Present address: Hoffmann–La Roche, Nutley, NJ 07110.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M36780).

formed by using the University of Wisconsin Genetics Computer Group software. Computer searches for sequences homologous to clone 1F3 were conducted in the GenBank data base.

Northern (RNA) Blot Analysis. RNA [total cellular or poly(A)$^+$, as indicated] was prepared by the guanidine thiocyanate method (19) and electrophoresed on 1.3% agarose gels containing 1.8% formaldehyde. After electrophoresis, gels were blotted onto nylon filters (Nytran; Schleicher & Schuell) in 20× SSC overnight. Filters were baked at 80°C under vacuum for 2 hr and subsequently hybridized to nick-translated 1F3 cDNA probes, as described for screening of the CT.4R(IL-4) cDNA library, except that ^{32}P-labeled cDNA probe was used at 2×10^6 cpm/ml.

The β- and κ-casein probes were obtained by PCR amplification of cDNA, prepared from total cellular mammary tissue RNA, by using synthetic oligonucleotide primers derived from the published sequences of these cDNAs. The γ-casein probe was the gift of J. Rosen (Baylor College of Medicine, Houston, TX).

RESULTS

We have recently described the generation of a cDNA library derived from a lymphokine-dependent murine CTL line, CT.4R, and the cloning and characterization of a lipase cDNA from that library (15). In an effort to identify other molecules that may be involved in CTL-mediated cytolysis, we present here the characterization of a second cDNA similarly isolated from the CTL library.

CT.4R cells are distinctive in that they grow equally well in either IL-2 or IL-4. They were derived from the IL-2-dependent cell line CT.EV by culture in IL-4 for 1 month (23).

A

	Probe		
	CT.4R(IL-4)	CT.EV(IL-2)	BW5147
Group I	+	-	-
Group II	-	+	-
Group III	+	+	-

B

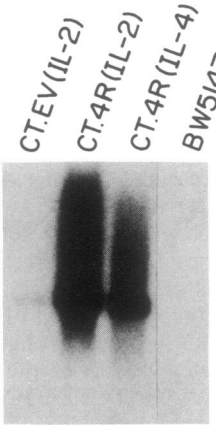

FIG. 1. Differential hybridization analysis of clone 1F3. (*A*) Hybridization patterns of clones in the CT.4R(IL-4) cDNA library. Approximately 10,000 clones were screened using ^{32}P-labeled cDNA probes derived from CT.EV(IL-2), CT.4R(IL-4), and BW5147 cells. The hybridization signals for each probe were scored, and clones were placed into one of three groups. (*B*) Hybridization pattern of clone 1F3. ^{32}P-labeled 1F3 cDNA was hybridized in Northern blot analysis to total cellular RNA from CT.EV(IL-2), CT.4R(IL-2), CT.4R(IL-4), and BW5147 cells. Each lane contained equivalent amounts of RNA, as determined by subsequent hybridization with a control actin probe.

A cDNA library was prepared from CT.4R cells grown in IL-4 and screened in triplicate by differential hybridization with cDNA probes prepared from CT.4R cells grown in IL-4, CT.EV cells grown in IL-2, and BW5147 thymoma cells. The hybridization pattern of one clone, 1F3, was characteristic of a group III clone (ref. 15 and Fig. 1*A*) and is shown in Fig. 1*B*. Clone 1F3 has a high level of expression in CT.4R cells grown either in IL-2 or in IL-4. Although clone 1F3 is expressed at somewhat lower levels in CT.EV cells, it is undetectable in BW5147 cells.

The complete nucleotide sequence and the deduced amino acid sequence of clone 1F3 is shown in Fig. 2. The original cDNA contained 1142 nucleotides, including a 3′-untranslated region of 440 nucleotides followed by a poly(A) tail of 9 bases. This poly(A) tail is preceded by a consensus polyadenylylation signal (AATAAA) (24), which is underlined in Fig. 2. The most 5′ 237 nucleotides of the full-length cDNA were obtained by PCR amplification of CT.4R cDNA, as described.

A comparison of the amino acid sequence encoded by clone 1F3 with sequences in the GenBank data base revealed a significant homology with the sequence for rat α-casein.

```
   1  ATGAAACTCCTCATCCTCACCTGCCTCGTGGCTGCTGCTTTTGCTATGCCCAGACTTCAT
 -15   M  K  L  L  I  L  T  C  L  V  A  A  A  F  A  M  P  R  L  H

  61  AGTAGAAATGCAGTTAGCAGTCAAACTCAGCAACAGCATAGCAGCAGTGAGGAAATTTTT
   6   S  R  N  A  V  S  S  Q  T  Q  Q  Q  H  (S) (S) (S) E  E  I  F

 121  AAACAACCAAAGTATCTCAACCTAAATCAGGAATTCGTCAACAACATGAACAGACAGAGA
  26   K  Q  P  K  Y  L  N  L  N  Q  E  F  V  N  N  M  N  R  Q  R

 181  GCACTTCTGACAGAACAGAACGATGAAATCAAGGTAACTATGGATGCAGCATCTGAGGAA
  46   A  L  L  T  E  Q  N  D  E  I  K  V  T  M  D  A  A  (S) E  E

 241  CAAGCAATGGCAAGTGCTCAGGAAGACTCTTCAATCAGCTCATCAAGTGAGGAATCTGAG
  66   Q  A  M  A  S  A  Q  E  D  S  (S) I  (S) (S) (S) (S) E  E  (S) E

 301  GAAGCTATTCCCAATATCACTGAGCAGAAAAACATTGCAAATGAAGACATGCTCAACCAG
  86   E  A  I  P  N  I  T  E  Q  K  N  I  A  N  E  D  M  L  N  Q

 361  TGCACCCTGGAACAGCTTCAGAGACAGTTTAAGTACAACCAACTTCTCCAGAAAGCTTCC
 106   C  T  L  E  Q  L  Q  R  Q  F  K  Y  N  Q [L  L  Q  K  A  S]

 421  CTGGCTAAGCAAGCTTCCCTGTTTCAGCAACCTTCCCTGGTACAGCAAGCTTCCCTGTTT
 126  [L  A  K  Q  A  S] [L  F  Q  Q  P  S] [L  V  Q  Q  A  S] [L  F

 481  CAGCAACCTTCCCTGCTTCAGCAAGCTTCCCTGTTTCAGCAACCTTCCATGGCTCAGCAA
 146  [Q  Q  P  S] [L  L  Q  Q  A  S] [L  F  Q  Q  P  S] [M  A  Q  Q

 541  GCTTCCCTGCTACAGCAACTTCTCCTGGCCCAGCAACCTTCCCTGGCACTGCAAGTTTCC
 166  [A  S] [L  L  Q  Q  L  L] [L  A  Q  Q  P  S] [L  A  L  Q  V  S]

 601  CCAGCACAGCAATCTTCCCTGGTACAGCAAGCTTTCCTGGCACAGCAAGCTTCCCTGGCA
 186  [P  A  Q  Q  S  S] [L  V  Q  Q  A  F] [L  A  Q  Q  A  S] [L  A

 661  CAGAAACATCATCCAAGACTGAGCCAGTCCTACTATCCACATATGGAACAGCCTTACAGA
 206  [Q  K  H  H] P  R  L  S  Q  S  Y  Y  P  H  M  E  Q  P  Y  R

 721  ATGAATGCATACAGCCAAGTTCAAATGAGACATCCTATGAGTGTAGTGGATCAGGCACTG
 226   M  N  A  Y  S  Q  V  Q  M  R  H  P  M  S  V  V  D  Q  A  L

 781  GCCCAGTTCTCTGTTCAGCCCTTCCCACAAATCTTCCAGTATGATGCCTTTCCCCTTTGG
 246   A  Q  F  S  V  Q  P  F  P  Q  I  F  Q  Y  D  A  F  P  L  W

 841  GCTTACTTTCCACAGGATATGCAGTACCTTACTCCCAAAGCTGTCCTTAACACCTTCAAG
 266   A  Y  F  P  Q  D  M  Q  Y  L  T  P  K  A  V  L  N  T  F  K

 901  CCCATTGTCTCCAAGGATACTGAAAAAACCAATGTGTGGTGAGATATTAAGACATCTCTC
 286   P  I  V  S  K  D  T  E  K  T  N  V  W  *

 961  AGGAACTCCACAGTTATAACATTTGATGTGACTGGACCCTCCATTCTCCATCCACCTCAT

1021  GTCTCTCTATTCATTCTATGGAGCCTCTGTTTTACCTAAAGGCTTGACTGCTATTTTAGA

1081  GTAGATAAAACACCATATAGAGGCCAATGATTCATCTTGAGTTGTCTACTGCATATTAGA

1141  TAGCATAGCATCCTTCCTTAAGCTAACCTTTCCTAGACAGATTCTTTTTTCTAAATTCCA

1201  GTTGCATCATCTGAGCGGAGAGAATCAAGGCCTTTAACAAGTTTCTATTATGGAAAATTT

1261  GTTTTAAAAGTCTTTAAATGGATTCTTCTGAAAATGTTATCATTTTGGGTAATTGTGTGT

1321  AGTAACTGAGATTGTCTTTTTCCTCTTTTCAATAAATTACATTTTAAGGCAAAAAAAAA
```

FIG. 2. Nucleotide and deduced amino acid sequences of the full-length clone 1F3 cDNA. Nucleotide positions are numbered at left. Translation of the open reading frame is shown below. The potential casein kinase phosphorylation sites are denoted by the circled residues. The repeated units described in the text are boxed. A consensus polyadenylylation signal is underlined. Star indicates translation termination.

```
mouse  MKLLILTCLVAAAFAMPRLHSRNAVSSQTQQQHSSSE..EIFKQPKYLNL  48
rat    MKLLILTCLVAAALALPRAHRRNAVSSQTQQENSSSEEQEIVKQPKYLSL  50

mouse  NQEFVNNMNRQRALLTEQNDEIKVTMDAASEEQAMASAQED.SSISSSSE  97
rat    NEEFVNNLNRQRELLTEQDNEIKITMDSSAEEQATASAQEDSSSSSSSSE 100

mouse  ESEEAIPNITEQKNIANEDMLNQCTLEQLQRQFKYNQLLQKASLAKQASL 147
rat    ESKDAIPSATEQKNIANKEILNRCTLEQLQRQIKYSQLLQQASLAQQASL 150

mouse  FQQPSLVQQASLFQQPSLLQQASLFQQPSMAQQASLLQQLLLAQQPSLAL 197
rat    ..........................AQQASLAQQALLAQQPSLAQ 170

mouse  QVSPAQQSSLVQQAFLAQQASLAQKHHPRLSQSYYPHMEQPYRMNAYSQV 247
rat    QAALAQQASLAQQASLAQQASLAQKHHPRLSQVYYPNMEQPYRMNAYSQV 220

mouse  QMRHPMSVVDQALAQFSVQPFPQIFQYDAFPLWAYFPQDMQYLTPKAVLN 297
rat    QMRHPMSVVDQ..AQFSVQSFPQLSQYGAYPLWLYFPQDMQYLTPEAVLN 268

mouse  TFKPIVSKDTEKTNVW* 314
rat    TFKPIAPKDAENTNVW* 285
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FIG. 3. Comparison of the amino acid sequence of the full-length clone 1F3 with that of rat α-casein. The deduced amino acid sequence of the full-length clone 1F3 is aligned with the amino acid sequence of rat α-casein, and residues identical in both sequences are boxed. Spacing (dots) is introduced to maximize alignment of the sequences. Translation of the open reading frame of clone 1F3 is shown with the first residue counted as position +1. The rat α-casein sequence is from ref. 25.

Fig. 3 shows the amino acid sequence of the full-length clone 1F3 compared with that of rat α-casein; identical residues are indicated. These two sequences are ≈81% identical overall when aligned with the spacing shown, whereas 14 of the first 15 residues, corresponding to the signal sequence of rat α-casein, are identical. Of particular importance is the presence of a six-residue-repeated motif distinctive for rodent α-caseins (25). Clone 1F3 contains a series of 15 repeated elements, shown boxed in Fig. 2, while 10 repeated elements are found in the rat casein sequence (25). In addition, clone 1F3 encodes a protein containing two clusters of casein kinase phosphorylation sites, -SXA-—where S is serine, X is any amino acid, and A is either glutamic acid or phosphoserine (26). Thus, there are 10 potential phosphoserine residues in clone 1F3 protein; these are denoted by the circled amino acids in Fig. 2.

The tissue distribution of clone 1F3 mRNA expression is shown in Fig. 4. High expression is seen in mammary tissue, as evidenced by the signal obtained after 4-hr exposure of a Northern blot of total cellular RNA from breast tissue. Although 1F3 mRNA expression was not detected in any other tissue examined by Northern analysis, low levels of 1F3 mRNA were seen in thymus after analysis by PCR amplification (data not shown).

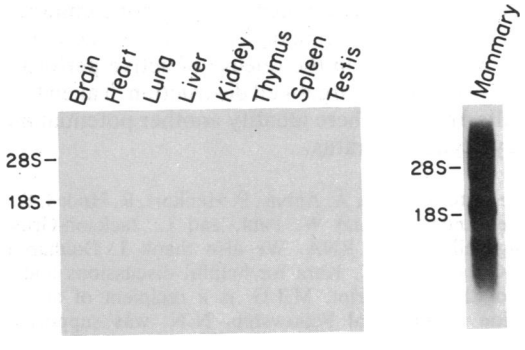

FIG. 4. Tissue distribution of 1F3 mRNA expression. ^{32}P-labeled 1F3 cDNA was hybridized in Northern blot analysis to ≈1 μg of poly(A)$^+$ RNA (all tissues except mammary) or 20 μg of total cellular RNA (mammary tissue). Exposure time for all lanes, except mammary tissue RNA, was 4 days at −70°C with an intensifying screen. Exposure time for the lane containing mammary tissue RNA was 1 hr at room temperature.

To determine the cell-type specificity of clone 1F3 mRNA expression, Northern blot analysis of many cell lines—including fibroblasts, macrophage/myelomonocytic cells, and cells from mastocytomas, B- and T-cell lymphomas—was conducted. A total of 26 cell lines examined failed to reveal clone 1F3 mRNA expression (data not shown). Clone 1F3 mRNA was, however, preferentially expressed in CTL. Fig. 5 shows that one of three long-term polyclonal CTL lines (bm10-37) and three of three CTL clones examined express clone 1F3 mRNA. The level of clone 1F3 mRNA expression in these cells was variable, however, as 5-day exposure of the Northern blot for bm10-37 and 2C cells was required to approximate the signal seen after 4-hr exposure of the blot for CT.4R(IL-2) and G8 clones. Other cytolytic cells, such as natural killer cells, as well as six of seven T cells of the T helper cell phenotype, all failed to reveal clone 1F3 mRNA expression. Low levels of expression have been noted in the T helper cell line D10.G4.1 and the Abelson virus-transformed pre-B cell line 2M3 (data not shown).

Finally, we examined whether other members of the casein gene family are also expressed in CTL. Fig. 6 shows that both clone 1F3 and β-casein are expressed in the CTL clones CT.4R(IL-2) and G8 but not in the thymoma BW5147 cell line. Interestingly, κ-casein is expressed in G8 but not in CT.4R(IL-2) clones, whereas γ-casein, a fourth member of

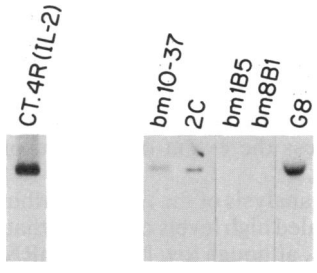

FIG. 5. Expression of clone 1F3 mRNA in CTL. ^{32}P-labeled 1F3 cDNA was hybridized in Northern blot analysis to ≈15 μg of total cellular RNA (all lanes except bm10-37) or 1 μg of poly(A)$^+$ RNA (bm10-37) from the indicated cells. bm10-37, bm1B5, and bm8B1 are polyclonal CTL lines; CT.4R(IL-2), 2C, and G8 are CTL clones. Exposure times are as indicated in text. Each lane contained equivalent amounts of RNA, as determined by subsequent hybridization with a control actin probe.

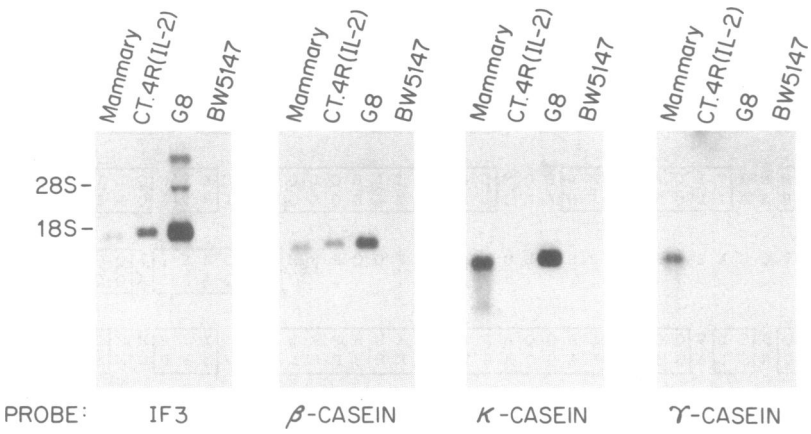

FIG. 6. Expression of casein genes in CTL. ^{32}P-labeled 1F3 cDNA or probes for β-, γ-, and κ-casein described in text were hybridized in Northern blot analysis to ≈2 μg of total cellular RNA from the indicated tissue and cell lines. Exposure times are 4 hr at room temperature (1F3 and β-casein) or 16 hr at −70°C (γ- and κ-casein).

the casein gene family, which is expressed in mammary tissue, is not expressed in either of these two CTL clones.

DISCUSSION

In this report we describe the isolation and characterization of a cDNA, the expression of which is restricted to cytotoxic T cells and mammary tissue. The complete amino acid sequence deduced from this cDNA clone is ≈81% identical to the previously reported sequence for rat α-casein (25). A comparison of the signal peptides of the full-length clone 1F3 and rat α-casein reveals that 14 of 15 residues are identical. In addition, this cDNA encodes a protein containing several casein kinase phosphorylation sites that are also found in rat α-casein. Furthermore, this cDNA contains 15 repeated elements of 18 nucleotides each, 10 of which are also found in the rat α-casein gene. These repeated units were predicted to be present in the mouse α-casein gene (25), given the larger apparent M_r of mouse α-casein in SDS/PAGE compared with other mammalian α-caseins (27). Given the above conservation of nucleotide sequence and protein structure, as well as its restricted expression, this cDNA most likely encodes mouse α-casein. Although a cDNA for mouse α-casein has been isolated from mammary tissue (27, 28), the sequence of this gene has not been reported. The sequences for other mouse members of this gene family, β-, ε-, and κ-casein, have been described (29–31).

α-Casein expression was not detected in Northern blot analysis of 26 hematopoietic cell lines. This gene was, however, isolated from a CTL library, and α-casein expression was noted in several CTL lines. Because casein expression may not be regulated solely at the transcriptional level, given that cytolytic effector molecules are often found preformed and stored in dense granules, mRNA for this gene might not be detected, even though functional protein is present. Thus, specific antisera to casein will be required to more fully address the extent of casein expression in CTL clones and lines.

Northern blot analysis of the tissue distribution of α-casein expression revealed high levels of mRNA that were limited to mammary tissue, although low levels of mRNA were seen in thymus after analysis by PCR amplification (data not shown). Interestingly, Lee *et al.* (32) have recently described the unexpected expression of a rat β-casein–chloramphenicol acetyltransferase fusion gene in the thymus of transgenic animals. Similarly, we have noted relatively high levels of endogenous β-casein expression in mouse thymus (data not shown). Although the cell lineage expressing either the transgene construct or endogenous α- and β-caseins has not been identified, the lineage could be CTL developing in the thymus. In support of that hypothesis, we have demonstrated that mature CTL that express α-casein may also express β- and κ-casein mRNAs.

How might caseins be involved in CTL function? Strong evidence now implicates perforin as an important CTL effector molecule. It is unclear, however, what other molecules are involved in the delivery of the "lethal hit." Although serine esterases have been colocalized in cytolytic granules with perforin (12, 13), alone they do not exhibit cytolytic activity (33). Nevertheless, several studies have shown the necessity for a proteolytic step in CTL-mediated lysis (34–37), one presumably involving serine esterases. Recently, serine esterases, which are secreted by activated CTL, have been shown to use casein as a substrate for proteolysis (17, 18).

One structural feature of caseins is their ability to form calcium-dependent micelles. Although calcium is required to maintain the integrity of the micelle, it is not present in the form of free calcium but rather as insoluble calcium phosphates (16). Given these observations, we propose that casein micelles act as a vehicle by which perforins are delivered onto the surface of target cells. Unlike conventional lipid vehicles, these protein micelles would not serve as substrates for polyperforin formation. Furthermore, the absence of free calcium within the micelle would prevent the premature polymerization of perforin monomers. Thus, upon activation, CTL would release perforin molecules enclosed within "casein cages" such that they are in an environment that maintains their cytolytic potential. The concomitant secretion and extracellular action of serine esterases on the casein micelles would result in the release of active perforin molecules and subsequent target-cell destruction. Taken together, the results presented here identify another potential member of the cytolytic apparatus.

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