Cloning of a Gene That Encodes a New Member of the Human Cytotoxic Cell Protease Family†,‡

M. Meier,§ P. C. Kwong,∥ C. J. Frêgeau,∥ E. A. Atkinson,∥ M. Burrington,∥ N. Ehrman,∥ O. Sorensen,∥ C. C. Lin,⊥ J. Wilkins,+ and R. C. Bleackley*,∥,○

Departments of Biochemistry, Immunology, Pathology, and Surgery, University of Alberta, Edmonton, Alberta, Canada T6G 2H7, and Departments of Medicine and Medical Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0V9

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ABSTRACT: A family of unusual serine proteases that are believed to be involved in the effector mechanism of cell-mediated cytotoxicity have previously been described in the mouse. However, in the human only one gene encoding a member has been isolated. By use of a mixture of murine cDNAs as probes, a second human gene has now been isolated. The primary structures of the gene and the predicted protein are very similar to those of the mouse. In addition, in keeping with the postulated involvement in cytolysis, transcripts were detected only in cytotoxic cells. The organization of the coding and noncoding regions of the gene, the clustering of family members, and the chromosomal location, close to the α chain of the T cell antigen receptor, are all conserved between human and mouse.

Cytotoxic T lymphocytes (CTL)[1] and natural killer cells (NK) are major effectors in cell-mediated cytotoxic reactions. They are responsible for the eradication of virally transformed cells and the rejection of transplanted organs. In addition they likely play a role in protection against tumors by lysing malignant cells. Over the past few years a number of molecules that are believed to play a role in the mechanism of cell-mediated cytotoxicity have been characterized (Podack, 1989).

Serine proteases have been implicated in CTL- and NK-mediated lysis (Pasternak & Eisen, 1985), and a family of cytotoxic cell protease (CCP) genes have been cloned and characterized from the mouse (Bleackley et al., 1988a,b). Transcripts corresponding to these genes are expressed specifically in cytolytic cells and accumulate during cytotoxic cell activation both in vitro and in vivo (Lobe et al., 1986; Mueller et al., 1988). Furthermore, the proteins encoded have been shown to be sequestered within the cytoplasmic granules, which are believed to carry potential effectors of cytolysis (Redmond et al., 1987). These facts together with the results that demonstrate abrogation of killing by serine protease inhibitors (Hudig et al., 1984, 1989) argue in favor of a key role for CCPs in the cytotoxic cell effector mechanism.

CCP1–CCP4 have identical and a somewhat unusual exon–intron organization, which sets them apart from other serine protease genes (Lobe et al., 1988). All four of these genes are clustered in the mouse genome on chromosome 14 close to the gene that encodes the α chain of the T cell antigen receptor (Crosby et al., 1990). In addition to the four murine protease genes that we have cloned and characterized, Weissman reported the cloning of the Hanukah factor (HF) gene (Gershenfeld & Weissman, 1986) and Tschopp (Masson & Tschopp, 1987) has described the members of the granzyme family. CCP1–CCP4 and HF correspond to granzymes B, C, E, F, and A, respectively. Despite the large number of murine members, only two genes that encode human counterparts, HF (Gershenfeld et al., 1988) and CCP1 (Schmid & Weissmann, 1987), have been described, and the amino-terminal sequence of a third polypeptide has been reported (Hameed et al., 1988). Although HF is clearly a granular protease, it has a lower level of homology than the others and is encoded on a different chromosome. The relationship of the third protease (Hameed et al., 1988) to the family is unclear at present and awaits further sequence data. Thus only one member of the human CCP family has been reported.

Here we report the cloning of a new human gene which on the basis of amino acid sequence, genomic organization, and chromosome location is a second member of this cytotoxic cell protease gene family.

Materials and Methods

Cell Culture. EL4.E1 is a subclone of a murine helper T cell lymphoma described by Farrar et al. (1980) which secretes high levels of interleukin 2 (IL2) when cultured in the presence of tumor-promoting phorbol esters. MTL2.8.2 is a cloned murine cytotoxic T cell line generated from CBA/J mice as described by Bleackley et al. (1982). Jurkat is a cloned human helper T-cell leukemia line which secretes high levels of IL2 on stimulation with T cell mitogens (Gillis & Watson, 1980). Lymphokine-activated killer (LAK) cells are human peripheral blood lymphocytes cultured in the presence of recombinant human IL2 (100 units/mL). Cytotoxic activity was determined in a chromium release assay as described previously (Bleackley et al., 1982).

All cells were maintained in RPMI 1640 media modified by the addition of 20 mM sodium bicarbonate, 0.34 mM sodium pyruvate, 0.02 M HEPES [*N*-(2-hydroxyethyl)-

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‡ The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J02907.

* Address correspondence to this author at the Department of Biochemistry, University of Alberta.

§ Department of Surgery, University of Alberta.

∥ Department of Biochemistry, University of Alberta.

⊥ Department of Pathology, University of Alberta.

+ Departments of Medicine and Medical Microbiology, University of Manitoba.

○ Department of Immunology, University of Alberta.

[1] Abbreviations: CTL, cytotoxic T lymphocytes; NK, natural killer cells; CCP, cytotoxic cell protease; HF, Hanukah factor; IL2, interleukin 2; LAK, lymphokine-activated killer.

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piperazine-*N'*-2-ethanosulfonic acid] adjusted to pH 7.3, 100 mM β-mercaptoethanol, and 10% (v/v) heat-inactivated fetal calf serum. In addition, the media was supplemented with 100 IU/mL penicillin G potassium and 100 μg/mL streptomycin sulfate. All cultures were incubated under 5% CO_2 in air, at 37 °C, 100% humidity, and were maintained in disposable plastic flasks.

Isolation of RNA. Total cellular RNA was recovered from cells [(1–5) × 10^6 cells] by homogenization of cell pellets in 3 mL of 4 M guanidinium thiocyanate, 0.5% sodium *N*-lauroylsarcosine, 25 mM sodium citrate (pH 7.0), and 0.1 M 2-mercaptoethanol. Homogenization was performed with a Polytron cell disruptor using three 30-s cycles with constant cooling on ice. The homogenate was then layered over 2 mL of 5.7 M cesium chloride and 0.1 M EDTA, pH 7.0, and the RNA was pelleted by centrifugation in a Beckman SW50.1 swinging-bucket rotor (36000 rpm × 12–20 h at 20 °C). The RNA pellet was quickly rinsed with ice-cold autoclaved distilled water to remove residual cesium and then was resuspended in at least 200 μL of 0.1% SDS and 25 mM EDTA with gentle heating (42 °C). The RNA was made 0.3 M in sodium acetate (pH 5.3) and precipitated by addition of 2.5 volumes of 95% ethanol and storage at −70 °C overnight. The pellet was collected by centrifugation (10000 rpm × 30 min) in a Sorvall SS34 rotor. The RNA pellet was then dried and resuspended in 10 mM Tris-HCl, pH 7.4, 1.0 mM EDTA, and 0.1% SDS. Lithium chloride was added to a final concentration of 0.5 M and poly(A+) RNA (mRNA) was isolated from this material by passage over an oligo(dT)–cellulose column (Collaborative Research). All solutions used in the isolation of RNA were treated with 0.1% diethyl pyrocarbonate to inhibit ribonuclease activity.

Gels and Blotting. RNA was separated on denaturing agarose gels and transferred to nylon membranes according to standard methods (Thomas, 1980). The running buffer consisted of 20 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.0 (MOPS), 5 mM sodium acetate, and 1.0 mM EDTA. The buffer was recirculated during electrophoresis as described by Maniatis et al. (1982). RNA samples (4 μg) were denatured by incubation at 55 °C for 15 min in running buffer containing 6.5% formaldehyde and 50% deionized formamide. After electrophoresis (140 V, 1.5 h) the RNA was transferred to nylon membranes (Hybond-N, Amersham) by capillary transfer. Following transfer, lane origins were marked, and the RNA was cross-linked to the nylon membranes by UV irradiation for 5–10 min.

Human placenta and thymus DNA (10 μg) was digested overnight with restriction enzyme (50 units, BRL). Following size fractionation on a 0.5% agarose gel at 30 V for 18 h, the DNA was transferred to a Hybond-N nylon membrane.

Nucleic Acid Probes and Hybridization Conditions. DNA fragments to be used as probes were prepared from recombinant plasmids that contained cDNAs corresponding to four murine CCP genes (Lobe et al., 1986; Bleackley et al., 1988a,b). The released *Eco*RI inserts were separated from vector DNA by electrophoresis through a 3.5% polyacrylamide gel. Gels were stained in ethidium bromide solution (0.5 μg/mL) and examined under short-wave UV transillumination. The band containing the desired DNA was then cut from the gel and the DNA purified by the "crush and soak" technique (Schleif & Wensink, 1981).

Purified DNA fragments were labeled by either nick translation or random priming with commercially available kits (BRL, Boehringer-Mannheim) and [α-^{32}P]dCTP (>3000 Ci/mmol). Labeled DNA was separated from unincorporated ^{32}P by exclusion chromatography.

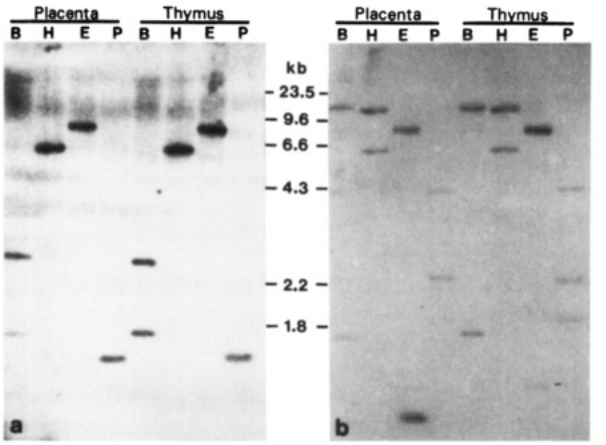


FIGURE 1: Southern blot of human genomic DNA. High molecular weight DNA from isolated human thymus and placental tissue. After overnight digestion with either *Eco*RI (E), *Bam*H1 (B), *Hin*dIII (H), or *Pst*1 (P), the DNA was fractionated by agarose gel electrophoresis, transferred to nylon membrane, and probed with human C158 (a) and human CCP1 (b) cDNAs. Double-stranded DNA size markers are indicated.

Prior to prehybridization, all membranes were washed in 5× SSC to remove residual salts from the transfer buffer. Filters for cross-species screening (mouse versus human) were prehybridized with gentle agitation at 42 °C for at least 60 min in a solution containing 20% formamide (v/v), 6× SSC, 5× Denhardt's solution, and 0.5% SDS; 1× Denhardt's solution contains 0.02% (w/v) each of ficoll, poly(vinylpyrrollidone), and bovine serum albumin. DNA probes were denatured by boiling for 5 min, quick cooled in ice, and then added to fresh prehybridization solution. Blots were incubated in the hybridization mix for a further 18–24 h. The concentration of ^{32}P-labeled probe in each hybridization was (0.5–4.0) × 10^6 cpm/mL.

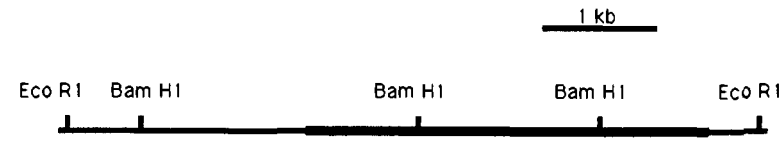
Following hybridization, membranes were washed twice (15 min each time) in 2× SSC and 0.1% SDS and twice in 1× SSC and 0.1% SDS at room temperature. More stringent hybridization conditions with 50% formamide solutions and washing in 2× SSC and 0.2× SSC at 45 or 55 °C were performed for intraspecies hybridizations (human versus human). Following washing, filters were wrapped in Saran Wrap and exposed to film (Kodak X-Omat AR).

Isolation of Genomic Sequences. A human placental genomic library, in λ Charon 4A, was plated out on *Escherichia coli* NEM 259 bacterial lawns to a density of approximately 25000–50000 plaques to each 150-mm plate. The plates were incubated at 37 °C until the plaques were confluent. Phage transfer to Hybond-N nylon filters was then performed in duplicate. The filters were then prehybridized, hybridized with ^{32}P-labeled DNA probes, washed, and exposed for autoradiography under the conditions described above.

After plaque purification phage DNA, prepared according to Davis et al. (1986), was digested with *Eco*RI in the presence of RNase A, prior to separation on 0.5% low-melt agarose gels. The *Eco*RI band representing the human genomic sequences of interest (as revealed by Southern blots) was then cut from the gel with a razor blade and saved for subsequent subcloning.

The gel fragment containing the genomic DNA insert was then prepared for subcloning by heating to 65 °C for 5 min. The liquid gel was then cooled to 37 °C, and then 8 μL of 5× ligase buffer, 50 ng of *Eco*RI-digested pUC13 dephosphorylated by calf intestinal alkaline phosphatase, and 4 units of T4 DNA ligase were added. The reaction was incubated at 14 °C for 18 h and then diluted to a 250-μL total volume with

a



b

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        10        20        30        40        50        60        70        80        90       100       110       120       130
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       140       150       160       170       180       190       200       210       220       230       240       250       260
ATTTTCCTCAATCTGAGCCCACCTCCCATTCCACACCCAGACTTATAAATCTGAGGCTAGATAGACACTCAGCAAAGATCGAAAAATGAAGGGTGTTCCCTAAAAGGTTTAATGGGTGTTAGCCTCTCCC

       270       280       290       300       310       320       330       340       350       360       370       380       390
TAGACCTCTCCTTTATGACCTGGAGTGTGGATTGTTCTTAGAAAGGCATTTGGTAGGGAATGTGAAGCTAAAAAAGATAAGTAATTATTACTCTACACTCCAACCCAGGAAAGAGGAGCTCAGACACCAA

       400       410       420       430       440       450       460       470       480       490       500       510       520
GTTGCAGTCATAGGAGTTGTTACTGGACTCAGCTTAAGAACCACTTATTCAGTGCCCCCACCCAGTCACCCCTGCCAGGAGGGAAGCCCATGGTGCAACTGATCTCAGAGATGGCAAGATGACTGTGTCC

       530       540       550       560       570       580       590       600       610       620       630       640       650
ATAGCTCTCCCATTACCTTGGCTCCACCTGGGCTTTGCGATTCATTTTTAGTTGATTTCTCCACTTCCTTCTGCCTTTGCAGGCCCTCAGCTACTCCACTGACCTGGTGATAACCCCCTCTAACATCCCT

       660       670       680       690       700       710       720       730       740       750       760       770       780
GAGGTCCTGAATCCCACCAGCACTACCCCCACTAAACCTCAGCCAAAGGCTAATTGGAGGCTATTCATTTATGCACCAAACAACACTTACTGAGAACCTAGAATGTGTTCAGCCCTGGCACATGAGAATT

       790       800       810       820       830       840       850       860       870       880       890       900       910
TTAGAAAATCCAACTCCAGAAGCTCATGGGTAGATCATTAGAAAATGGCACCAATCAGGAGGACAGCAGAGGCTCAGAGGAATCAGAGAGACTCACTAAAAAGGAGGAGCCTGGGAAGTCCTGTCCAGGA

       920       930       940       950       960       970       980       990      1000      1010      1020      1030      1040
TCCTCTGGGGCTCTACCACTGTGAAAATAGATCTTTCTTCCTCCATGTGTGTTTCTGCAGTAGGATGCAGAAGTCAAGCACTGTCCATTCTTTCTGAGAGAAGGTTCTGAGACTTAAAGTTCAGAAAAGC

      1050      1060      1070      1080      1090      1100      1110      1120      1130      1140      1150      1160      1170
TTGCTCTGCCTGCAAGGAGTACTGCCTGCAGAGCTCAGGAGGACCCCTCCCTCACTGTCTGCAGCACACAAAACACTTCACCCTCAATCACTGTCGTCCTAAGCCTTCTCTCCTGACTGCAGCCATCCCC

      1180      1190      1200      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
AGCTACTTTATCTTCCAGTCTTTCCTTTCAGAGGAGATCATCGGGGGCCATGAGGCCAAGCCCCACTCCCGCCCCTACATGGCCTTTGTTCAGTTTCTGCAAGAGAAGAGTCGGAAGAGGTGTGGCGGCA

      1310      1320      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430
TCCTAGTGAGAAAGGACTTTGTGCTGACAGCTGCTCACTGCCAGGGAAGGTAAGGAGCAGCACCAGCTCACCTCCTGAGTTCCCCCTACAGGGACCCTTGTTTTCTCCTGGGGACTGCAGCCCAGGGGAG

      1440      1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550      1560
CTTCCAGAGTTCCTGGTAACACAAGCCCATGAAAGCTCATCGGAGCAGCCACCTGGGAATAGGACTAAGATGATGAAAGGCTGAGAAATAGGACAGGGTCAGAGGGTCAGAGGGTCAGAGGTCAGAGCAA

      1570      1580      1590      1600      1610      1620      1630      1640      1650      1660      1670      1680      1690
GTGGCATGGTTTCACACACTGGCCCAATAAGGCAGTAGTAAAGGTTGAATCCATGCATTAAGGATCAAAATGCAAACTCGAGCAATTTCATGATATTTCCTGAAGGCAAGAGAGAGCCCCCAGAAGCCCT

      1700      1710      1720      1730      1740      1750      1760      1770      1780      1790      1800      1810      1820
ACTCTTCATGTCTTTGTGAAGTAGAGGGCACAGTCACTCAGCCCTGGAGCCCTCCTGTCCCTTCAACTTCCCCTCAGCTGCAGCCCTACCCTGCCACAGCTGTCATCTGCTCTTCACTGCTCCTGGGCTC

      1830      1840      1850      1860      1870      1880      1890      1900      1910      1920      1930      1940      1950
TATCCCCTGTGACTCCACCCCCATCCTCACTCTGCTCTCTGTGCAGCTCCATAAATGTCACCTTGGGGGCCCACAATATCAAGGAACAGGAGCGGACCCAGCAGTTTATCCCTGTGAAAAGACCCATCCC

      1960      1970      1980      1990      2000      2010      2020      2030      2040      2050      2060      2070      2080
CCATCCAGCCTATAATCCTAAGAACTTCTCCAACGACATCATGCTACTGCAGGTGAGGCACACTCCTGCCACTCTTGCTCTTCTTGGTCCAGTTGGTTCCACTCCCACTGGGATGCCGGCCCTTTCCTCC

      2090      2100      2110      2120      2130      2140      2150      2160      2170      2180      2190      2200      2210
TTTCCATCCTGACCTCTTGGTCAGTTCCTGTGCCTTAGAGGAGAGGGAAGATTGTGCAGCCCCATCACTGTGTCGGGGCCCAGAAGCCATTGCCTGACCTGGACTTTCTTGCTTCTTCCCCACCAGCTG

      2220      2230      2240      2250      2260      2270      2280      2290      2300      2310      2320      2330      2340
GAGAGAAAGGCCAAGTGGACCACAGCTGTGCGGCCTCTCAGGCTACCTAGCAGCAAGGCCCAGGTGAAGCCAGGGCAGCTGTGCAGTGTGGCTGGCTGGGGTTATGTCTCAATGAGCACTTTAGCAACCA

      2350      2360      2370      2380      2390      2400      2410      2420      2430      2440      2450      2460      2470
CACTGCAGGAAGTGTTGCTGACAGTGCAGAAGGACTGCCAGTGTGAACGTCTCTTCCATGGCAATTACAGCAGAGCCACTGAGATTTGTGTGGGGGATCCAAAGAAGACACAGACCGGTTTCAAGGTTGG

      2480      2490      2500      2510      2520      2530      2540      2550      2560      2570      2580      2590      2600
GTTTCCAGCCTCTACCCAGAAAGACCACAGGGAGAGAGGAACTTGAGGGAGTTCTGGGGTATGACAGTGGCCAGATCTTTATGCTCTCAGCCCAGAGCTTGGGCAGCCTGTGTCCCCCTGGAATCTAGTC

      2610      2620      2630      2640      2650      2660      2670      2680      2690      2700      2710      2720      2730
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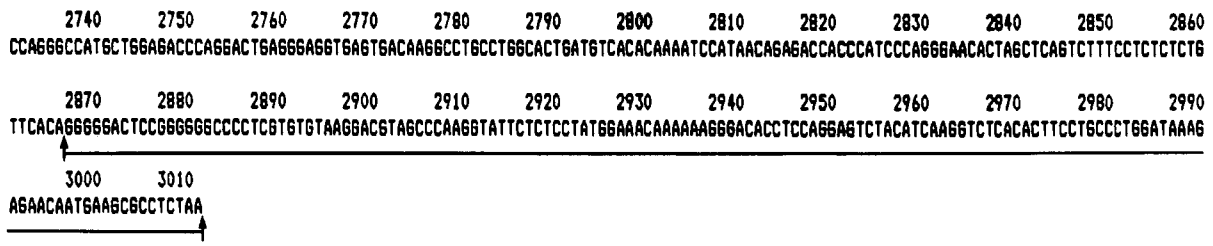

C

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                                30                                  60                                  90
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Met Gln Pro Phe Leu Leu Leu Leu Ala Phe Leu Leu Thr Pro Gly Ala Gly Thr Glu Glu Ile Ile Gly Gly His Glu Ala Lys Pro His

                               120                                 150                                 180
TCC CGC CCC TAC ATG GCC TTT GTT CAG TTT CTG CAA GAG AAG AGT CGG AAG AGG TGT GGC GGC ATC CTA GTG AGA AAG GAC TTT GTG CTG
Ser Arg Pro Tyr Met Ala Phe Val Gln Phe Leu Gln Glu Lys Ser Arg Lys Arg Cys Gly Gly Ile Leu Val Arg Lys Asp Phe Val Leu

                               210                                 240                                 270
ACA GCT GCT CAC TGC CAG GGA AGC TCC ATA AAT GTC ACC TTG GGG GCC CAC AAT ATC AAG GAA CAG GAG CGG ACC CAG CAG TTT ATC CCT
Thr Ala Ala His Cys Gln Gly Ser Ser Ile Asn Val Thr Leu Gly Ala His Asn Ile Lys Glu Gln Glu Arg Thr Gln Gln Phe Ile Pro

                               300                                 330                                 360
GTG AAA AGA CCC ATC CCC CAT CCA GCC TAT AAT CCT AAG AAC TTC TCC AAC GAC ATC ATG CTA CTG CAG CTG GAG AGA AAG GCC AAG TGG
Val Lys Arg Pro Ile Pro His Pro Ala Tyr Asn Pro Lys Asn Phe Ser Asn Asp Ile Met Leu Leu Gln Leu Glu Arg Lys Ala Lys Trp

                               390                                 420                                 450
ACC ACA GCT GTG CGG CCT CTC AGG CTA CCT AGC AGC AAG GCC CAG GTG AAG CCA GGG CAG CTG TGC AGT GTG GCT GGC TGG GGT TAT GTC
Thr Thr Ala Val Arg Pro Leu Arg Leu Pro Ser Ser Lys Ala Gln Val Lys Pro Gly Gln Leu Cys Ser Val Ala Gly Trp Gly Tyr Val

                               480                                 510                                 540
TCA ATG AGC ACT TTA GCA ACC ACA CTG CAG GAA GTG TTG CTG ACA GTG CAG AAG GAC TGC CAG TGT GAA CGT CTC TTC CAT GGC AAT TAC
Ser Met Ser Thr Leu Ala Thr Thr Leu Gln Glu Val Leu Leu Thr Val Gln Lys Asp Cys Gln Cys Glu Arg Leu Phe His Gly Asn Tyr

                               570                                 600                                 630
AGC AGA GCC ACT GAG ATT TGT GTG GGG GAT CCA AAG AAG ACA CAG ACC GGT TTC AAG GGG GAC TCC GGG GGG CCC CTC GTG TGT AAG GAC
Ser Arg Ala Thr Glu Ile Cys Val Gly Asp Pro Lys Lys Thr Gln Thr Gly Phe Lys Gly Asp Ser Gly Gly Pro Leu Val Cys Lys Asp

                               660                                 690                                 720
GTA GCC CAA GGT ATT CTC TCC TAT GGA AAC AAA AAA GGG ACA CCT CCA GGA GTC TAC ATC AAG GTC TCA CAC TTC CTG CCC TGG ATA AAG

AGA ACA ATG AAG CGC CTC TAA
Arg Thr Met Lys Arg Leu End
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FIGURE 2: Primary sequence analysis of the human protease gene. (a) Restriction map of genomic fragment. Region sequenced indicated by heavier line. (b) Nucleotide sequence of genomic fragment. Underlined regions correspond to exons. (c) Predicted cDNA and protein sequences.

TE. Competent DH5α *E. coli* cells were then transformed with 50 μL of the diluted ligation reaction. Colorless colonies were then selected for plasmid DNA isolation, and the identity of the inserts was confirmed by Southern blotting as previously described. By use of a combination of overlapping restriction and deletion fragments and specific oligonucleotide priming, the sequence of both strands was determined by the chain termination method (Sanger et al., 1977).

cDNA Isolation. Double-stranded cDNA was synthesized from 2 μg of human LAK cell poly(A+) RNA with a cDNA synthesis kit (Boehringer-Mannheim). The double-stranded cDNA was methylated with *Eco*RI methylase, and then *Eco*RI linkers were attached. Size selection was carried out on a Sepharose 4B column. After ligation into λ Zap arms (Stratagene), the cDNA was packaged into phage with Gigapack Gold packaging extract.

A portion of the library (480 000 pfu) was not amplified and plated directly onto 150-mm NZY agar plates (40 000 pfu per plate) and after 18-h incubation at 37 °C transferred onto 137-mm Hybond-N nylon filters. Two copies of each plate were made. After denaturation in 0.5 M NaOH and 1.5 M NaCl and neutralization in 1.0 M Tris-HCl, pH 8, and 1.5 M NaCl, the filters were baked at 80 °C for 1.5 h. The filters were hybridized with a 1.5-kb *Bam*H1 genomic fragment (above) as described earlier.

Positive plaques were identified by autoradiography at room temperature overnight. Agar plugs (0.5 cm) containing positive plaques were removed and soaked in SM (0.1 M NaCl, 4 mM $MgSO_4$, 20 mm Tris-HCl, pH 7.5, 2% gelatin). Eluted phage were then screened again at a lower density so that positive clones were represented by single-well isolated plaques. Agar plugs of 1.5 mm containing the single isolated clone were removed and soaked in SM. Bluescript rescue (Stratagene) was performed to switch the cloned cDNAs from λ Zap into the bluescript phagemid and transformed into *E. coli* BB4.

In Situ Hybridization. Purified cDNA insert was isolated from an agarose gel after *Eco*RI digestion and labeled with [^{3}H]dCTP by random primer extension to a specific activity of 1×10^8 cpm/μg. About 1.5 ng/slide of ^{3}H-labeled DNA probe was used (1.5×10^5 cpm) with chromosome preparations that were 3–4 weeks old. Hybridization was performed in 50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 2× SSC, and sonicated salmon sperm DNA (0.42 mg/mL) at 37 °C for 16 h. The slides were then washed in five changes of

50% formamide–2× SSC at 41 °C, 2 min each, followed by five changes of 2× SSC at 41 °C for 2 min each and dehydrated in 70%, 80%, 90%, and 100% ethanol. A detailed procedure of in situ hybridization, autoradiography, and chromosome identification has been described previously (Linn et al., 1985).

Results and Discussion

We believe that the cytotoxic cell proteases play a key role in the mechanism by which CTL and NK cells destroy foreign cells (Bleackley et al., 1988a,b). Therefore, it would be of great interest to study the expression of the human protease genes in pathological situations of clinical relevance. A number of laboratories have attempted to clone the genes that encode these human proteases; however, only one member of the CCP family and the human analogue of HF have so far been reported (Gershenfeld et al., 1988). Indeed, three different groups independently cloned the same human CCP1 gene from cDNA libraries of activated cytotoxic cells (Schmid & Weissmann, 1987; Caputo et al., 1988; Trapani et al., 1988). We reasoned that perhaps other members were present at very low frequency in these libraries and were therefore missed. Indeed, preliminary Southern blot data with human genomic DNA suggested to us that there were other related genes present. In addition, the amino-terminal sequence of a third esterase from human LAK cells has been reported (Hameed et al., 1988).

Isolation of a Human CCP Gene. In order to isolate the human homologues, a human genomic library was screened by hybridization, at reduced stringency, with a mixture of the murine genes (Lobe et al., 1986; Bleackley et al., 1988a,b) as a probe. Phage DNA from one of the positive plaques was particularly interesting as it gave a 6.3-kb *Eco*RI fragment (and ultimately a 1.5-kb *Bam* fragment) that hybridized with the murine probes at low stringency, but when it was probed with human CCP1 cDNA (R. C. Bleackley, unpublished results), only weak cross-hybridization was detected under high-stringency conditions. This 1.5-kb genomic fragment was used to screen a LAK cell cDNA library, and one strongly hyridizing sequence was detected and isolated. When this cDNA, designated C158, was used as a probe for a Southern blot of human genomic DNA, a different pattern (Figure 1a) was obtained from that seen when human CCP1 cDNA was hybridized with the blot (Figure 1b). From this result we concluded that C158 represented a different gene from human CCP1. Preliminary sequence analysis confirmed that C158 and the 1.5-kb genomic fragment came from the same gene and, moreover, that the protein encoded was highly homologous to the murine CCP family but clearly different from human CCP1. The Southern blot data shown in Figure 1 also indicate that neither gene is rearranged upon commitment to the T cell lineage.

The restriction map of the 6.3-kb *Eco*RI genomic fragment is shown in Figure 2a. The nucleotide sequence of the region indicated by the heavy line is presented in Figure 2b. A comparison of this sequence with those of the murine CCP genes (Lobe et al., 1988) revealed high levels of homology (~70% identity) in regions that corresponded to exons and dissimilarity within introns. By placing the introns in exactly the same places that they occur in the murine sequences (all four murine genes have introns in precisely the same positions) (Lobe et al., 1988), the sequence of a cDNA could be determined (Figure 2c). The sequence of the partial cDNA (C158) isolated is identical with residues 347–741 in Figure 2c.

The Protein Encoded by the New Gene Does Not Have a Murine Counterpart. The predicted protein that would be encoded by this gene is 246 amino acids in length (M_r 27318) and is shown in Figure 2c below the nucleotide sequence. This protein was not found in the GenBank data base; however, it is homologous to a wide variety of serine proteases. The highest level of identity was with the cytotoxic cell proteases (human 70%, murine 61%), cathepsin G (human 57%), and mast cell proteases (40–45%). In addition a significant level of identity (~30%) was found with many other trypsin- and chymotrypsin-like enzymes. Clearly this protein is a new member of the serine protease superfamily and will subsequently be referred to as h-CCPX (see below for explanation).

An alignment of the sequence with those predicted from the murine genes (Figure 3a) illustrates the high degree of amino acid sequence similarity and also reveals that h-CCPX shares many features in common with the other CCP's (Bleackley et al., 1988a,b). It is very basic (14% basic/6% acidic amino acids) and contains a hydrophobic leader sequence of 18 residues followed by a putative zymogen dipeptide (residues 19 and 20) that precedes the mature protease amino-terminal Ile residue. It is believed that the basic nature of these proteins may play a role in sequestering them within granules bound to proteoglycans (Stevens et al., 1988). The two sequences +21 to +24 (Ile-Ile-Gly-Gly) and +29 to +36 (Pro-His-Ser-Arg-Pro-Tyr-Met-Ala), which are found in all the CCPs, granzymes, RMCPI and II, and cathepsin G, are also conserved in the new protease as are the six cysteine residues which form disulfide bonds (Jenne & Tschopp, 1988). The catalytic triad residues (*) that form the active site of the serine proteases are all present in the correct positions (Neurath, 1984). The sequences surrounding these, which are highly conserved in serine proteases, are also conserved. We noted in the initial report of the sequence of CCP1 and CCP2 that both contained unusual residues in regions that are believed to be important in defining substrate specificity (Lobe et al., 1986; Murphy et al., 1988). In addition they all lack a disulfide bond that in other serine proteases is important in restricting the size of the substrate binding pocket. Similar results were subsequently found for the other CCP's and granzymes (Bleackley et al., 1988a,b; Masson & Tschopp, 1987). The protese described here also has unusual residues in these same sites and lacks the disulfide bond. However, the pattern of amino acids seen in this protein (Figure 3b), namely, Thr, Ser-Tyr-Gly, and Gly at positions −6, +15 to +17, and +25 relative to the active site Ser, does not correspond to any of the murine proteases characterized to date. Consequently, even though this protease corresponds to the second member of the human family, it would be confusing to give it the suffix 2. The definition of its substrate specificity will be useful in deciding if it is the human analogue of one of the murine proteases. However, for the time being it will be called h-CCPX.

h-CCPX Is Expressed in Cytotoxic Cells. Transcription of the murine CCP genes is induced in cytolytic cells when they are activated. Using total RNA from activated human cells, it proved to be difficult to demonstrate this for h-CCPX due to the low abundance of mRNA. Therefore, poly(A+) RNA was purified from resting and activated peripheral blood lymphocytes and subjected to Northern blot analysis using the 1.5-kb genomic fragment as a probe (Figure 4). In the left-hand panel a transcript is clearly present in the activated cells that is absent in RNA from the unstimulated control. sometimes we see a small amount of transcript in the unstimulated lane perhaps due to cellular contamination; however, the transcript is always induced upon stimulation. In contrast to the human HF transcript that was shown to be present in

a

```
                                                                                                  *
h-CCPX   1   Met Gln Pro Phe Leu Leu Leu Leu Ala Phe Leu Leu Thr Pro Gly Ala Gly Thr Glu Glu Ile Ile Gly Gly His Glu Ala Lys Pro His Ser Arg Pro Tyr Met Ala Phe Val Gln
m-CCP1   1   Met Lys Ile Leu Leu Leu Leu Leu Thr Leu Ser Leu Ala Ser Arg Thr Lys Ala Gly Glu Ile Ile Gly Gly His Glu Val Lys Pro His Ser Arg Pro Tyr Met Ala Leu Leu Ser
m-CCP2   1   Met Pro Pro Val Leu Ile Leu Leu Thr Leu Leu Leu Pro Leu Arg Ala Gly Ala Glu Glu Ile Ile Gly Gly Asn Glu Ile Ser Pro His Ser Arg Pro Tyr Met Ala Tyr Tyr Glu Phe
m-CCP3   1   Met Pro Pro Val Leu Ile Leu Leu Thr Leu Leu Leu Pro Leu Gly Ala Gly Ala Glu Glu Ile Ile Gly Gly His Val Val Lys Pro His Ser Arg Pro Tyr Met Ala Phe Val Lys Ser
m-CCP4   1   Met Pro Pro Ile Leu Ile Leu Leu Thr Leu Leu Leu Pro Leu Arg Ala Gly Ala Glu Glu Ile Ile Gly Gly His Glu Val Lys Pro His Ser Arg Pro Tyr Met Ala Arg Val Arg Phe

                                                                                         *
h-CCPX   40  Phe Leu Gln Glu Lys Ser Arg Lys Arg Cys Gly Gly Ile Leu Val Arg Lys Asp Phe Val Leu Thr Ala Ala His Cys Gln Gly Ser Ser Ile Asn Val Thr Leu Gly Ala His Asn Ile
m-CCP1   40  Ile Lys Asp Gln Gln Pro Glu Ala Ile Cys Gly Gly Phe Leu Ile Arg Glu Asp Phe Val Leu Thr Ala Ala His Cys Glu Gly Ser Ile Ile Asn Val Thr Leu Gly Ala His Asn Ile
m-CCP2   41  Leu Lys Val Gly Gly Lys Lys Met Phe Cys Gly Gly Phe Leu Val Arg Asp Lys Phe Val Leu Thr Ala Ala His Cys Lys Gly Ser Ser Met Thr Val Thr Leu Gly Ala His Asn Ile
m-CCP3   41  Val Asp Ile Glu Gly Asn Arg Arg Tyr Cys Gly Gly Phe Leu Val Gln Asp Asp Phe Val Leu Thr Ala Ala His Cys Arg Asn Arg Thr Met Thr Val Thr Leu Gly Ala His Asn Ile
m-CCP4   41  Val Lys Asp Asn Gly Lys Arg His Ser Cys Gly Gly Phe Leu Val Gln Asp Tyr Phe Val Leu Thr Ala Ala His Cys Thr Gly Ser Ser Met Arg Val Ile Leu Gly Ala His Asn Ile

                                                                                                         *
h-CCPX   80  Lys Glu Gln Glu Arg Thr Gln Gln Phe Ile Pro Val Lys Arg Pro Ile Pro His Pro Ala Tyr Asn Pro Lys Asn Phe Ser Asn Asp Ile Met Leu Leu Gln Leu Glu Arg Lys Ala Lys
m-CCP1   80  Lys Glu Gln Glu Lys Thr Gln Gln Val Ile Pro Met Val Lys Cys Ile Pro His Pro Asp Tyr Asn Pro Lys Thr Phe Ser Asn Asp Ile Met Leu Leu Lys Leu Lys Ser Lys Ala Lys
m-CCP2   81  Lys Ala Lys Glu Glu Thr Gln Gln Ile Ile Pro Val Ala Lys Ala Ile Pro His Pro Asp Tyr Asn Pro Asp Asp Arg Ser Asn Asp Ile Met Leu Leu Lys Leu Val Arg Asn Ala Lys
m-CCP3   81  Lys Ala Lys Glu Glu Thr Gln Gln Ile Ile Pro Val Ala Lys Ala Ile Pro His Pro Asp Tyr Asn Ala Thr Ala Phe Phe Ser Asp Ile Met Leu Leu Lys Leu Glu Ser Lys Ala Lys
m-CCP4   81  Arg Ala Lys Glu Glu Thr Gln Gln Ile Ile Pro Val Ala Lys Ala Ile Pro His Pro Ala Tyr Asp Asp Lys Asp Asn Thr Ser Asp Ile Met Leu Leu Lys Leu Glu Ser Lys Ala Lys

h-CCPX   120 Trp Thr Thr Ala Val Arg Pro Leu Arg Leu Pro Ser Ser Lys Ala Gln Val Lys Pro Gly Gln Leu Cys Ser Val Ala Gly Trp Gly         Tyr Val Ser Met Ser Thr Leu Ala Thr
m-CCP1   120 Arg Thr Arg Ala Val Arg Pro Leu Asn Leu Pro Arg Arg Asn Val Asn Val Lys Pro Gly Asp Val Cys Tyr Val Ala Gly Trp Gly     Arg Met Ala Pro Met Gly Lys Tyr Ser Asn
m-CCP2   121 Arg Thr Arg Ala Val Arg Pro Leu Asn Leu Pro Arg Arg Asn Ala His Val Lys Pro Gly Asp Glu Cys Tyr Val Ala Gly Trp Gly     Lys Val Thr Pro Asp Gly Glu Phe Pro Lys
m-CCP3   121 Arg Thr Lys Ala Val Arg Pro Leu Lys Leu Pro Arg Pro Asn Ala Arg Val Lys Pro Gly Asp Val Cys Ser Val Ala Gly Trp Gly Ser Arg Ser Ile Asn Asp Thr Lys Ala Ser Ala
m-CCP4   121 Arg Thr Lys Ala Val Arg Pro Leu Lys Leu Pro Arg Pro Asn Ala Arg Val Lys Pro Gly His Val Cys Ser Val Ala Gly Trp Gly Arg Thr Ser Ile Asn Ala Thr Gln Arg Ser Ser

h-CCPX   158 Thr Leu Gln Glu Val Leu Leu Thr Val Gln Lys Asp Cys Gln Cys Glu Arg Leu Phe His Gly Asn Tyr Ser Arg Ala Thr Glu Ile Cys Val Gly Asp Pro Lys Lys Thr Gln Thr Gly
m-CCP1   159 Thr Leu Gln Glu Val Glu Leu Thr Val Gln Lys Asp Arg Glu Cys Glu Ser Tyr Phe Lys Asn Arg Tyr Asn Lys Thr Asn Gln Ile Cys Ala Gly Asp Pro     Lys Thr Lys Arg Ala
m-CCP2   160 Thr Leu His Glu Val Lys Leu Thr Val Gln Lys Asp Gln Val Cys Glu Ser Gln Phe Gln Ser Ser Tyr Asn Arg Ala Asn Glu Ile Cys Val Gly Asp     Ser Lys Ile Lys Gly Ala
m-CCP3   161 Arg Leu Arg Glu Ala Gln Leu Val Ile Gln Glu Asp Glu Glu Cys Lys Lys Arg Phe Arg His     Tyr Thr Glu Thr Thr Glu Ile Cys Ala Gly Asp Leu Lys Lys Ile Lys Thr Pro
m-CCP4   161 Cys Leu Arg Glu Ala Gln Leu Ile Ile Gln Lys Asp Lys Glu Cys Lys Lys Tyr Phe Tyr     Lys Tyr Phe Lys Thr Met Gln Ile Cys Ala Gly Asp Pro Lys Lys Ile Gln Ser Thr

                                 *
h-CCPX   198     Phe Lys Gly Asp Ser Gly Gly Pro Leu Val Cys Lys Asp Val Ala Gln Gly Ile Leu Ser Tyr Gly Asn Lys Lys Gly Thr Pro Pro Gly Val Tyr Ile Lys Val Ser His Phe Leu
m-CCP1   198 Ser Phe Arg Gly Asp Ser Gly Gly Pro Leu Val Cys Lys Lys Val Ala Ala Gly Ile Val Ser Tyr Gly Tyr Lys Asp Gly Ser Pro Pro Arg Ala Phe Thr Lys Val Ser Ser Phe Leu
m-CCP2   199 Ser Phe Glu Glu Asp Ser Gly Gly Pro Leu Val Cys Lys Arg Ala Ala Ala Gly Ile Val Ser Tyr Gly Gln Thr Asp Gly Ser Ala Pro Gln Val Phe Thr Arg Val Leu Ser Phe Val
m-CCP3   200     Phe Lys Gly Asp Ser Gly Gly Pro Leu Val Cys Asp Asn Lys Ala Tyr Gly Leu Leu Ala Tyr Ala Lys Asn Arg Thr Ile Ser Ser Gly Val Phe Thr Lys Ile Val His Phe Leu
m-CCP4   200     Tyr Ser Gly Asp Ser Gly Gly Pro Leu Val Cys Asn Asn Lys Ala Tyr Gly Val Leu Thr Tyr Gly Leu Asn Arg Thr Ile Gly Pro Gly Val Phe Thr Lys Val Val His Tyr Leu

h-CCPX   237 Pro Trp Ile Lys Arg Thr Met Lys Arg Leu
m-CCP1   238 Ser Trp Ile Lys Lys Thr Met Lys Ser Ser
m-CCP2   239 Ser Trp Ile Lys Lys Thr Met Lys His Ser
m-CCP3   239 Pro Trp Ile Ser Arg Asn Met Lys Leu Leu
m-CCP4   239 Pro Trp Ile Ser Arg Asn Met Lys Leu Leu
```

b

	-6	+15 → +17	+25
h-CCPX	Thr	Ser Tyr Gly	Gly
h-CCP1	Thr	Ser Tyr Gly	Arg
m-CCP1	Ala	Ser Tyr Gly	Arg
m-CCP2	Ala	Ser Tyr Gly	Gln
m-CCP3	Thr	Ala Tyr Ala	Gly
m-CCP4	Ser	Thr Tyr Gly	Gly

FIGURE 3: Comparison of human and mouse cytotoxic cell proteases. (a) Alignment of h-CCPX primary amino acid sequence with the murine CCPs using MicroGenie. The catalytic triad amino acids are indicated by (*). The signal sequence and putative activation peptides correspond to residues 1–18 and 19–20, respectively. (b) Comparison of substrate binding pocket residues between the CCP family. The numbers define residues relative to the active site Ser.

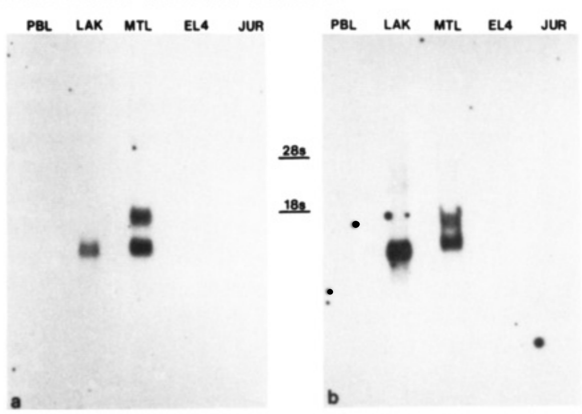

FIGURE 4: Northern blot probed with h-CCPX cDNA. Poly(A+) RNA was isolated from resting (PBL) and interleukin 2 activated (LAK) human peripheral blood lymphocytes, a murine cytotoxic T cell clone (MTL), and human (JUR) and murine (EL4) interleukin 2 producing T cells. After electrophoresis and transfer to nylon, the blots were hybridized with a cDNA corresponding to h-CCPX. Washing was at 45 (a) and 55 °C (b) in 0.2× SSC. The exposure times for autoradiography were 2 and 12 days, respectively.

the human helper cell line Jurkat (Gershenfeld et al., 1988), h-CCPX transcripts were not detected in this T helper line.

Because of the high level of homology between the various family members, cross-hybridization can occur. In the case of the murine genes, CCP1 can be distinguished from the others because of a difference in transcript size (Lobe et al., 1986). However, the transcripts detected by h-CCP1 and h-CCPX are very similar in electrophoretic mobility. Therefore, high-stringency washing conditions were established under which cross-hybridization was minimized. Thus in panel a (washed at 45 °C) the probe detects transcripts in both human and mouse cytotoxic cells. However, at 55 °C the signal due to the cross-hybridization with the mouse transcripts is markedly less than that seen for the human RNA, even though this mouse cell line expresses extremely high levels of the protease transcripts. The human–human and human–mouse nucleotide identities are all approximately 70%; thus, the signal seen in the RNA from activated human cells is due to specific hybridization with h-CCPX transcripts.

In addition to the data shown here no detectable signal was detected with this probe on RNA samples from a number of human cell lines obtained from ATCC including CEM-CM3, CCRF-CEM, and CCRF-SB (acute lymphoblastic leukemias), RPMI 7666 (EBV-transformed B lymphoblast), DLD-1 (co-

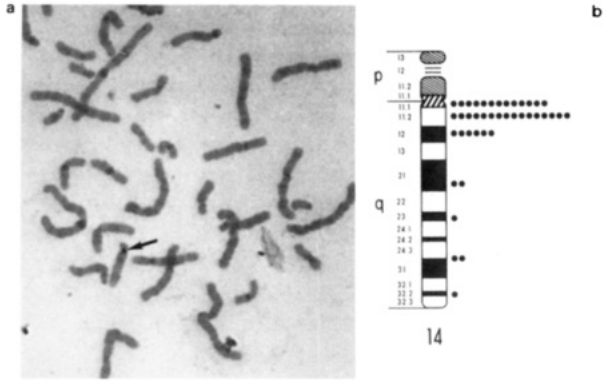

FIGURE 5: Localization of the ESP gene to chromosome 14. (a) A representative partial metaphase of a normal male after in situ hybridization with a labeled h-CCPX cDNA probe (C158). The arrow indicates a silver grain over the 14q11.1→11.2 region of chromosome 14. (b) Idiogram of chromosome 14 showing the distribution of 41 grains clustered in the region q11.1→q12.

lon, adenocarcinoma), CRL-7020 (thymus), CRL-7123 (spleen), and freshly isolated human splenocytes and thymocytes.

The h-CCPX Gene Maps to Chromosome 14. It was initially shown that gene CTLA-1 (identical with CCP1 and granzyme B) was located on murine chromosome 14 close to the α chain of the T cell antigen receptor locus (Brunet et al., 1986). Subsequently, the human CTLA-1 gene was localized to human chromosome 14 again close to TCR-A (Harper et al., 1988). We recently confirmed the location of murine CCP1 and in addition demonstrated that three other family members (CCP2, -3, and -4) were also present there (Crosby et al., 1990). It was therefore of considerable interest to determine the chromosomal location of the new human protease as it clearly is part of the same family as the cytotoxic cell proteases.

Purified insert from the cDNA containing plasmid (C158) was labeled by random priming and used as a probe for in situ hybridization with 115 human metaphase spreads as described previously (Lin et al., 1985). The total number of silver grains in these metaphase spreads was 171 with an average of 1–2 grains per cell. Of the total grains scored, 41 were found on chromosome 14 (24%) (Figure 5a). The remaining grains appeared to be randomly distributed on other chromosomes without a significant hybridization region. Among those grains located on chromosome 14, 35 (85%) were clustered on the region 14q11.1→q12 with a peak at q11.1→q11.2 (Figure 5b). It should be noted that, under the conditions used for in situ hybridization, cross-hybridization with the h-CCP1 gene cannot be ruled out; however, no other significant localization of grains was observed. These results indicate that the new human protease gene is likely located on chromosome 14 in the proximal region of 14q11.1→q11.2. This is exactly the same region of chromosome 14 where human CCP1 is located (Harper et al., 1988). It is intriguing that the linkage of the CCP family of genes with the T cell antigen receptor α chain locus is conserved in mouse and human. Both loci are expressed exclusively in T lymphocytes. Perhaps they are under the influence of some common regulatory elements that control expression of this region of the genome.

The clustering of the CCP genes and the similarity of their coding sequences and their genomic organization indicate that they have evolved from a common ancestral gene. It is interesting that another related gene, that encoding human cathepsin G, has also been mapped to this same region (Hohn et al., 1989). Perhaps cathepsin G also evolved from this same primordial sequence. However, this protease is expressed in neutrophils and monocytes rather than lymphocytes. Thus we have the unusual situation of a family of closely related genes all present in the same region of the genome but expressed in different cell types. It will be most interesting to study the molecular basis for this differential expression.

In summary, the cloning of a new human serine protease gene is described. On the basis of sequence homology, genomic organization, chromosome localization, and cell-specific expression, it encodes a member of the cytotoxic cell protease family. The primary structure of the predicted protein resembles those of members of the murine family but is not convincingly the human counterpart of any one (perhaps its murine homologue remains to be discovered). Thus, human cytotoxic cells, like those of the mouse, express a number of unusual proteases that are delivered to the target cell by granule-mediated exocytosis after engagement of the antigen receptor. Why should so many proteases be involved? It is tempting to speculate that a protease cascade analogous to that seen in the complement system, and ultimately resulting in cell lysis, is involved (Reid, 1986). Alternatively, the proteases could recognize and cleave a group of similar but subtly different substrates as part of the killing mechanism. The resolution of this question will come only when the physiological substrates have been identified. With the genes in hand it is now possible to design experiments to address this intriguing problem. Finally, the cloning of the human CCP genes will allow studies on the expression of this family of genes in patients and thus provide extremely important information concerning the molecular basis of immune function/dysfunction and disease progression.

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Articles

Olfactory Transduction: Cross-Talk between Second-Messenger Systems†

Robert R. H. Anholt* and Ann M. Rivers
Department of Neurobiology, Box 3209, Duke University Medical Center, Durham, North Carolina 27710
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ABSTRACT: Chemosensory cilia of olfactory receptor neurons contain an adenylate cyclase which is stimulated by high concentrations of odorants. Cyclic AMP produced by this enzyme has been proposed to act as second messenger in olfactory transduction. Here we report that olfactory cilia contain calmodulin and that calmodulin potently activates olfactory adenylate cyclase by a mechanism additive to and independent from direct stimulation by odorants. Activation by calmodulin is calcium dependent and enhanced by GTP. Thus, olfactory transduction may involve a second-messenger cascade in which an odorant-induced increase in intracellular calcium concentration leads to activation of adenylate cyclase by calmodulin.

Olfaction takes place after air-borne odorants partition into the nasal mucus and interact with chemosensory cilia that protrude from the dendritic tips of olfactory receptor neurons (Getchell et al., 1984; Lancet, 1986; Anholt, 1989). Isolated olfactory cilia provide a preparation of chemosensory dendritic membranes thought to contain at least some, if not all, of the membrane-associated components that mediate odorant recognition and olfactory transduction (Anholt et al., 1986; Chen et al., 1986). Indeed, ciliary membrane preparations contain an odorant-sensitive adenylate cyclase (Pace et al., 1985; Sklar et al., 1986; Shirley et al., 1986; Pfeuffer et al., 1989) and its associated stimulatory GTP-binding protein (Pace et al., 1985; Anholt et al., 1987), G_{olf}, an olfactory neuron specific variant of G_s (Jones & Reed, 1989), and also odorant-gated cation channels (Labarca et al., 1988). The olfactory adenylate cyclase is stimulated by high concentrations of some, but not all, odorants (Pace et al., 1985; Sklar et al., 1986; Shirley et al., 1986), and cyclic AMP has, therefore, been proposed to act as second messenger in olfaction (Pace et al., 1985; Sklar et al., 1986; Shirley et al., 1986; Lancet & Pace, 1987; Nakamura & Gold, 1987; Gold & Nakamura, 1987). However, the enzyme is stimulated mostly by hydrophobic odorants at concentrations close to their aqueous solubility limits (Sklar et al., 1986; Anholt, 1987). Moreover, the same odorants at

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* To whom correspondence should be addressed.

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