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Novel Serine Proteases Encoded by Two Cytotoxic T Lymphocyte–Specific Genes

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Genes that are expressed exclusively in cytotoxic T cells should encode proteins that are essential for target cell lysis in cell-mediated immune responses. The sequences of two cytotoxic T lymphocyte–specific complementary DNA's (cDNA's) suggest that the two genes encode serine proteases. A full-length cDNA corresponding to one of the genes was isolated and sequenced. The predicted protein resembles serine proteases in that it includes all the residues that form the catalytic triad of the active site of serine proteases. Moreover, it has sequence characteristics thought to occur only in rat mast cell protease type II. These results are in accord with the view that a protease cascade plays a key role in cytotoxic T-cell activation.

CYTOTOXIC T LYMPHOCYTES (CTL's), also referred to as T killer cells, are effector cells in cell-mediated immune reactions. They specifically recognize foreign antigens on the surface of target cells, bind to them, and cause the target cells to lyse. Although the various steps in this process have been analyzed in considerable detail (*1, 2*), most studies have not provided insight into the mechanism by which the killer cell effects the lysis of a target cell. A means of identifying relevant molecules, based on cloning CTL-specific genes, has been described (*3*). The transcripts corresponding to two of these genes (B10 and C11) were detected exclusively in activated CTL's (*4*). Moreover, the kinetics of messenger RNA (mRNA) expression, as detected by these two cloned probes, closely paralleled but preceded cytotoxicity throughout cytotoxic responses in vitro (*3*).

Sequence analysis (*5*) of B10 and C11 (Fig. 1A) revealed that they were related to each other and that the hypothetical proteins they encode contain a short region characteristic of serine proteases, Asp-Ser-Gly-Gly (a sequence homologous to that surrounding Ser^{195} of chymotrypsin). With B10 and C11 as probes, another CTL complementary DNA (cDNA) library was screened, in which inserts greater than 1000 base pairs were cloned in λgt10. Forty thousand recombinants were screened and 39 plaques corresponding to C11 were isolated, but no evidence for a B10 recombinant could be found.

A cDNA insert of 1400 base pairs, which hybridized with C11, was selected for sequence analysis (*5*). The predicted protein sequence encoded, of molecular weight 25,319, is shown in Fig. 1B. The putative start codon is preceded by a potential ribosome binding site CCUUCCG (*6*), and a polyadenylation signal sequence AAUAAA (*7*) occurs just upstream from the poly(A) tract. Of the first 12 amino acids predicted, ten are hydrophobic, and the amino acid in position 2 (Lys) is basic, suggesting that this sequence may act as a signal to direct secretion or intracellular organelle location (*8*).

A search of the National Biomedical Research Foundation (NBRF) protein sequence data bank revealed that the protein encoded by C11 resembles a number of serine proteases (Table 1). When the se-

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quences were optimally aligned according to the Dayhoff algorithm (*9*), the homologies generally varied between 30 and 40 percent. The greatest homology was found with rat mast cell protease type II (RMCPII), which had amino acids identical to 109 of 215 amino acids encoded by C11, giving a match per length of 51 percent. The amino acid residues known to form the catalytic triad of the active site in serine proteases (His^{57}, Asp^{102}, and Ser^{195}) (*10*) were all found in the protein encoded by C11. The sequences around these residues, which are highly conserved among serine proteases, are also conserved in the C11 gene product. Indeed, largely because of conservation around this region, the protein encoded by C11 appears to be somewhat homologous (about 30 percent of 209 residues) even to the prokaryotic proteases trypsin and type B from *Streptomyces griseus*.

The cytotoxic T cell–specific proteins (CCP's) encoded by C11 and B10 will be referred to as CCPI and CCPII, respectively. In Fig. 2 the optimal protein alignment with CCPI is presented for RMCPII, bovine chymotrypsin, bovine trypsin, and CCPII. RMCPII is an intracellular serine protease found in the granules of atypical mast cells (*11*). The high level of homology of CCPI with RMCPII is particularly intriguing as RMCPII has a number of structural features that make it exceptional in the serine protease superfamily (*12*). Protein CCPI contains cysteines in precisely the same positions as RMCPII which, by analogy with RMCPII, form three disulfide bonds. These occur in the same positions in chymotrypsin, trypsin, and elastase. Both CCPI and RMCPII lack a disulfide bond that is present in all other known serine proteases, including several from prokaryotes (*13*), and that links Cys^{191} with Cys^{220} in chymotrypsin. In both CCPI and RMCPII the first of these two half-cystines is replaced by a phenylalanine, while the second half-cystine has been deleted along with other residues. Linkage of Cys^{191} to Cys^{220} is thought to be important in stabilizing the conformation of the substrate binding site (*12*). Its absence in CCPI and RMCPII may lead to significant changes in that site and, hence, in substrate specificity.

Two other primary structure changes previously seen only in RMCPII and thought to alter substrate binding are also present in the predicted CCPI protein. In RMCPII and CCPI the amino acid six residues before the active-site serine is alanine. In chymotrypsin-like proteases it is serine and in trypsin-like proteases, aspartic acid. The residue in this position lies at the bottom of the S_1 binding site (*14*), so the change to a less polar residue would indicate a preference for a hydrophobic amino acid at the P_1 position in the substrate (*13*). Furthermore, the sequence Ser-Trp-Gly^{216} in chymotrypsin, which forms hydrogen bonds with the P_1 and P_3 residues of the substrate (*14*), is replaced by Ser-Tyr-Gly in CCPI and RMCPII, again suggesting altered substrate specificity. Both of these changes are also seen with CCPII.

One of the few RMCPII-specific differences that is not present in CCPI is the substitution of isoleucine at position 99 in chymotrypsin for asparagine. In most mammalian serine proteases this residue is hydrophobic, and indeed in CCPI it appears to be phenylalanine. However, most of the RMCPII-specific changes are present in CCPI protein, suggesting that the substrate binding site of CCPI will resemble that of

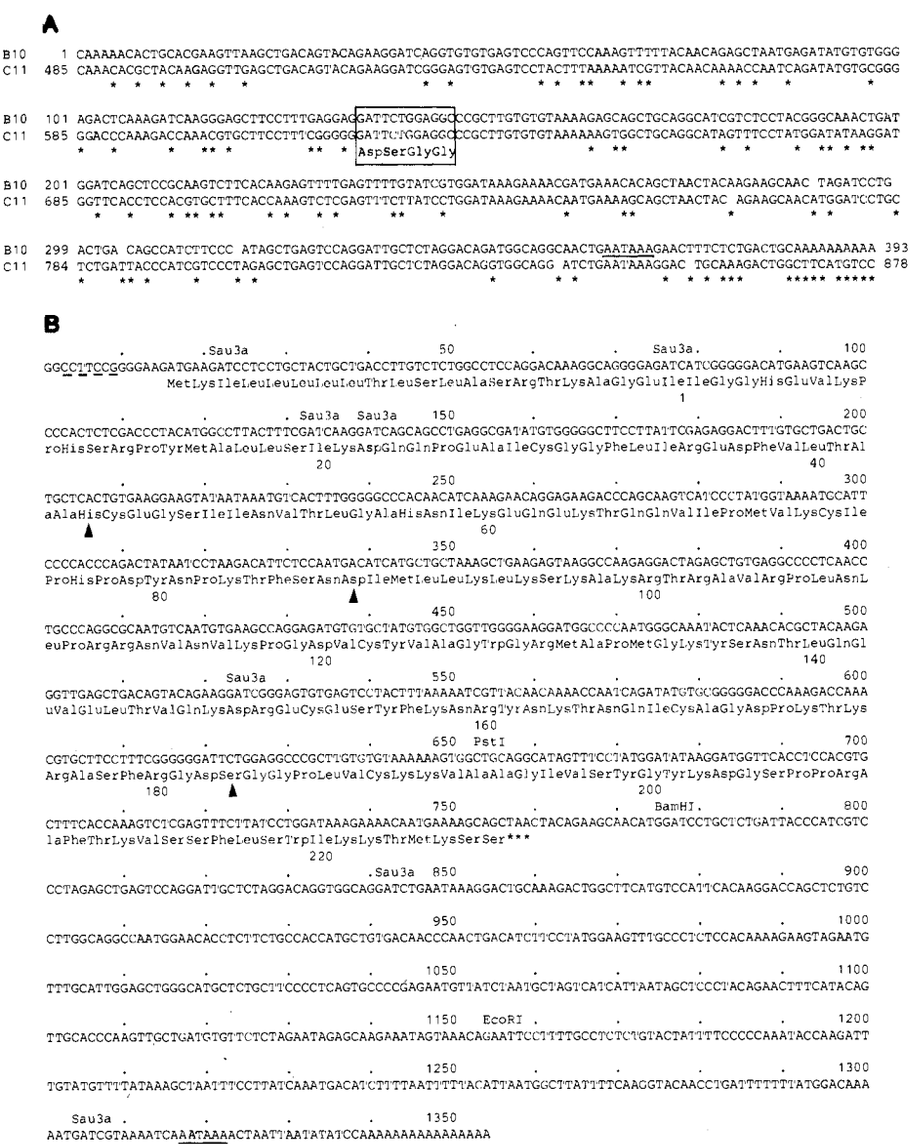

Fig. 1. (A) Nucleotide sequence comparison of clones B10 and C11. Insert DNA was purified from the CTL-specific clones designated B10 and C11 (*3*), recloned in M13 or pUC vectors and sequenced by the dideoxy method (*5*). The two sequences were maximally aligned; * designates nucleotide mismatches. The sequence of B10 is numbered from the first nucleotide of the insert, whereas that of C11 has been renumbered to correspond with the full-length sequence shown in (B). The region encoding the characteristic serine protease sequence Asp-Ser-Gly-Gly is boxed, and the polyadenylation signal sequence of B10 is underlined. (B) Nucleotide and predicted protein sequence of C11 insert. A size selected cDNA library (>1000 base pairs) was screened with a C11 insert isolated from the original library (*3*). A plaque that contained a cross-hybridizing sequence, whose apparent length was 1400 base pairs was selected for further analysis. Restriction fragments were subcloned in M13 or pUC13 and sequenced as above. A potential initiator methionine codon is present at nucleotide 16 followed by an open reading frame to nucleotide 756. A putative ribosome binding site (------) and a polyadenylation signal (——) are underlined. By analogy with RMCPII (see text and Fig. 2), the numbering of the C11 protein starts at the isoleucine 21 residues downstream from the proposed translational initiation site. The amino acids which form the catalytic triad of the serine proteases (His^{57}, Asp^{102}, and Ser^{195} in chymotrypsin) are marked (▲).

Table 1. A selection of proteins that are homologous to the predicted C11 protein, CCPI. The protein sequence predicted from the longest open reading frame encoded by CCPI was compared with the National Biomedical Research Foundation protein sequence data bank. The numbering for CCPI (see text) is given in Fig. 1B. The data bank numbering system has been used for homologous proteins. All of the proteins that were significantly homologous (>30%) with CCPI over a large portion of the molecule (>150 residues) were serine proteases.

Protein	E.C. number	Species source	Residues compared		Percent homology
			CCPI	Bank protein	
7S nerve growth factor	3.4.21	Murine	29-224	26-229	40
Chymotrypsin A	3.4.21.1	Bovine	1-200	16-216	35
Chymotrypsin B	3.4.21.1	Bovine	1-200	16-216	36
Complement C1r	3.4.21.41	Human	52-224	56-238	35
Elastase	3.4.21.11	Porcine	3-220	3-233	33
Factor X	3.4.21.6	Bovine	1-225	192-421	33
RMCPII	3.4.21	Rat	1-214	1-213	51
Kallikrein	3.4.21.8	Rat	26-225	51-262	36
Plasminogen	3.4.21.7	Human	3-224	563-787	37
Plasminogen activator	3.4.21.31	Human	72-224	389-560	35
Trypsin	3.4.21.4	*S. griseus*	29-220	22-214	33
Trypsin	3.4.21.4	Rat	29-226	31-228	39

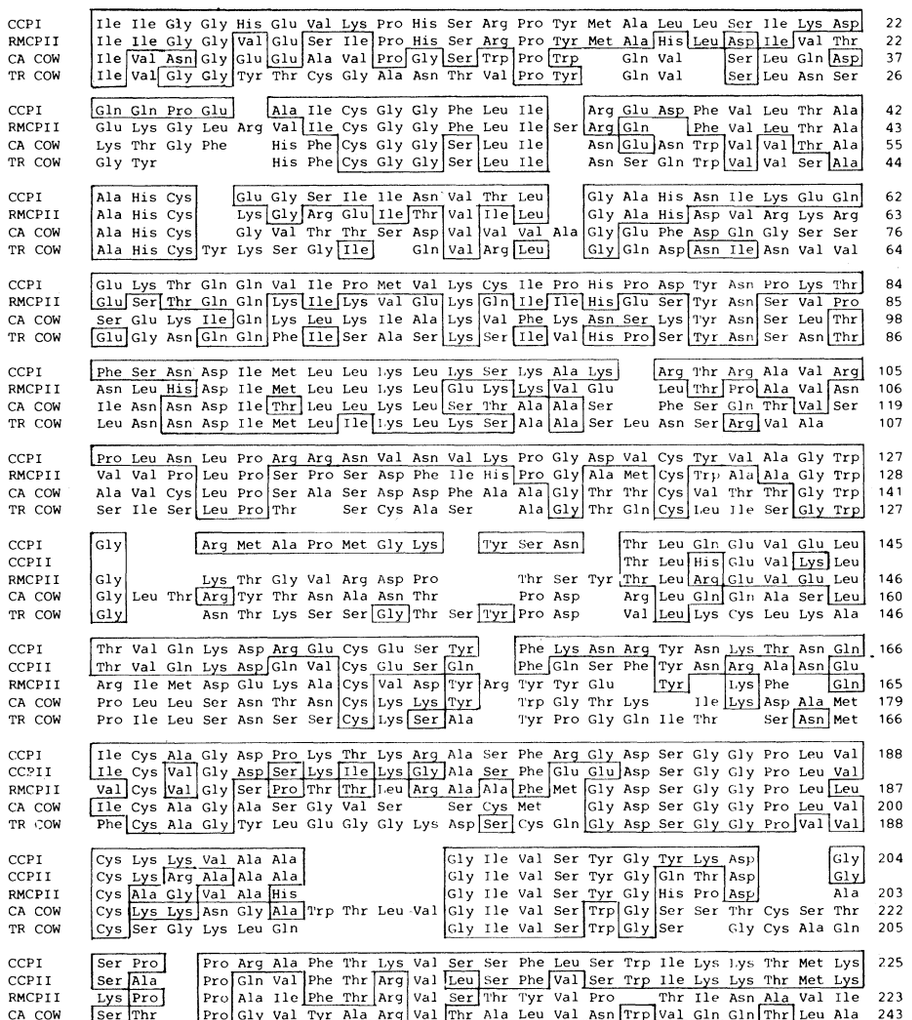

Fig. 2. Alignment of the predicted CCPI protein sequence with other serine proteases. The protein sequence predicted from C11 is aligned, according to the method of Dayhoff (*9*), with rat mast cell protease (RMCPII), bovine chymotrypsin (CA COW), bovine trypsin (TR COW), and the partial protein sequence predicted from insert B10 (CCPII). Amino acids that are identical to those of CCPI are boxed. Residue numbers are given at the end of each line; CCPI and RMCPII start at the Ile residue, whereas the numbering of CA and TR is based on the inactive zymogens. No numbering is presented for CCPII because the full sequence has yet to be determined.

RMCPII and could be significantly different from those of other mammalian serine proteases (*15*).

In addition to the interesting aspects of the structure of CCPI, the fact that both CCPI and CCPII appear to be serine proteases has significant implications for the mechanism by which CTL's are activated to become capable of lysing target cells (*1, 2*). Protease inhibitors have been shown to block T-cell effector function (*16*), and a trypsin-like esterase activity was shown to be induced during CTL activation (*17*). We have demonstrated that two genes that encode protease-like proteins are expressed in CTL's. Previous evidence that these genes are activated specifically in CTL's and that their expression correlates with cytolytic activity (*3*) suggests that they play a key role in the development of a cytotoxic response.

The two genes we have so far characterized appear to encode serine esterases, and another CTL-specific gene, AR10, cloned by Gershenfeld and Weissman (*18*) encodes a trypsin-like enzyme. These findings suggest that a protease cascade mechanism may be involved in T-cell cytotoxicity. Such a mechanism has been shown in a number of biological systems, including blood coagulation and complement-mediated lysis, and is advantageous in control, specificity, and amplification of enzymatic reactions (*19*).

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The Locale Map of Honey Bees: Do Insects Have Cognitive Maps?

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Whereas higher vertebrates are able to construct a mental "map" of their home area and so use their knowledge of the spatial relations between landmarks to navigate along novel routes, invertebrates have been thought able to use landmarks in their navigation only as a familiar, route-specific series. Experiments with honey bees show that these insects have and use landmark maps thus invalidating this presumed invertebrate-vertebrate dichotomy.

ALTHOUGH HONEY BEES USE CELESTIAL cues in their navigation to unfamiliar food sources (*1*), they frequently begin to rely on prominent landmarks, at least on the outward journey, as the route becomes familiar (*2, 3*). Wehner (*4*) has proposed that the landmark memory of invertebrates is stored as a series of route-specific photographs; hence, bees and other insects could use landmarks only in serial order and along familiar routes. This model stands in sharp contrast to the way higher vertebrates are thought to use landmarks as part of a map; in this system, the relative location of familiar landmarks is understood—presumably stored in the brain as a map—so that novel routes based on new combinations of landmarks may be used, freeing the animal from dependence on route-specific combinations.

To put this presumed vertebrate-invertebrate dichotomy into concrete terms, consider the area around an animal's home (Fig. 1). If the hive is its home and site A is a food source, an insect would be presumed to know site A in terms of a set of landmarks regularly encountered there; if displaced to site B, the insect would, at best, recognize site B as part of some other route leading from home to another food source, and perhaps be able to follow that route back home. A higher vertebrate familiar with the same area, on the other hand, would be able to use the landmarks visible at site B to determine the direction of site A, and set off directly toward it even though it had never traveled from site B to site A before; the vertebrate, it is supposed, has integrated its landmark knowledge into a map.

Two observations suggest that the map alternative might be available to bees. First, older foragers captured while leaving the hive, transported in darkness to a location hundreds of meters away and out of sight of the hive, fed a highly concentrated sugar solution, and released can fly directly home (*5, 6*); that young foragers cannot suggests that some familiarity with the locale may be important. This result is not conclusive since the foragers might have recognized the release site as part of their route-specific memory.

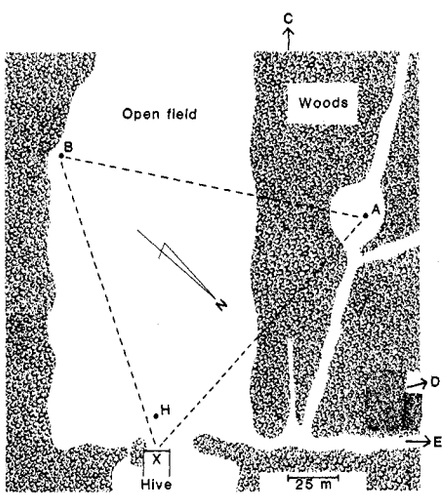

Fig. 1. Bees from a hive (bottom center) were trained to either site A, site B (both 160 m from the hive), or (off the top of the map) site C (350 m from the hive); on subsequent days these foragers and others were captured and transported to a different site and released—site B for those trained to A, site A for those trained to B, and site D (350 m from the hive) or site E (4425 m from the hive) for those trained to site C. As a control, some trained foragers were released at site H as well. Arrow N marks north.

The second suggestion comes from experiments in which forager dances were manipulated to indicate a location either in the middle of a lake or on the far shore (*7, 8*). Recruitment was effective only to the latter, more plausible site, suggesting that the dance coordinates enabled recruits to make some sort of judgment about the suitability of the advertised site before leaving the hive; recruits make economic judgments about the distance versus the quality of the food being advertised before leaving (*9*). But the lake results could be explained on the basis of environmental factors (*8*). The purpose of the experiments reported here was to determine whether bees are limited to route-specific navigation or, instead, have a true locale map available to them.

Individually marked honey bees *(Apis mellifera ligustica)* were first trained to site A 160 m west of the hive (Fig. 1) along a path through the woods at Stony Ford, the Princeton University field station in Princeton, New Jersey. On the basis of the training procedure and the observed departure bearings from both the hive and the food source, foragers flew either directly to and from the hive to reach the feeding station or along a two-legged route that included the last portion of the training route (the path). All bees visiting the station were either marked or captured.

On subsequent days, foragers regularly visiting site A were captured at the hive entrance in a 600-ml beaker placed in their takeoff path. Each trapped forager was then carried in the dark across an open field to site B, 160 m south-southwest of the hive, and released. Two foragers were sometimes captured and transported together, but bees

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