Serine proteinase inhibitors (serpins) are classically regulators of extracellular proteolysis, however, recent evidence suggests that some function intracellularly. Such “ovalbumin” serpins include the human proteinase inhibitors 6 (PI-6), 8 (PI-8), and 9 (PI-9), plasminogen activator inhibitor 2, and the monocyte/neutrophil elastase inhibitor. PI-9 is a potent granzyme B (graB) inhibitor that has an unusual P1 Glu and is present primarily in lymphocytes. In a search for the murine equivalent of PI-9 we screened cDNA libraries, and performed reverse transcriptase-polymerase chain reaction on RNA isolated from leukocyte cell lines and from lymph nodes and spleens of allo-immunized mice. We identified 10 new ovalbumin serpin sequences: two resemble PI-8, two resemble PI-9, and the remaining six have no obvious human counterparts. By RNA analysis only one of the two sequences resembling PI-9 (designated SPI6) is present in mouse lymphocytes while the other (a partial clone designated mBM2A) is predominantly in testis. SPI6 comprises a 1.8-kilobase cDNA encoding a 374-amino acid polypeptide that is 68% identical to PI-9, mBM2A is 65% identical to PI-9 and over 80% identical to SPI6. Although the reactive loops of SPI6 and mBM2A differ from PI-9, both contain a Glu in a region likely to contain the P$_1$-P$_1'$ bond. SPI6 produced in vitro using a coupled transcription/translation system formed an SDS-stable complex with human graB and did not interact with trypsin, chymotrypsin, leukocyte elastase, pancreatic elastase, thrombin, or cathepsin G. Recombinant SPI6 produced in a yeast expression system was used to examine the interaction with human graB in more detail. The second-order rate constant for the interaction was estimated as $8 \times 10^4$ M$^{-1}$ s$^{-1}$, and inhibition depended on the Glu in the SPI6 reactive center. The SPI6 gene was mapped to the same region on mouse chromosome 13 as SpI3, which encodes the murine homolog of PI-6. We conclude that even though their reactive centers are not highly conserved, SPI6 is a functional homolog of PI-9, and that the regulation of graB in the mouse may involve a second serpin encoded by mBM2A. Our identification of multiple sequence homologs of PI-8 and PI-9, and six new ovalbumin serpins, is consonant with the idea that the larger set of granule and other proteinases known to exist in the mouse (compared with human) is balanced by a larger array of serpins.

Serine proteinase inhibitors (serpins) form a superfamily of proteins that resemble $\alpha_1$-proteinase inhibitor in structure (1). The majority regulate proteinase activity in extracellular processes such as fibrinolysis, blood coagulation, complement activation, and tissue remodeling (2). Serpins generally contain an exposed C-terminal loop in which the reactive center P$_1$-P$_1'$ residues define the inhibitory specificity of the molecule. Inhibition of proteolysis is achieved by the formation of essentially irreversible 1:1 stoichiometric complexes between serpins and cognate proteinases (2, 3).

Chicken ovalbumin is the prototype of a branch of the serpin superfamily (4). These ovalbumin serpins are grouped by amino acid sequence similarity, and the lack of N- and C-terminal sequences present in $\alpha_1$-proteinase inhibitor. They include plasminogen activator inhibitor (PAI-2 (5)),$^1$ monocyte/neutrophil elastase inhibitor (MNEI (6)), squamous cell carcinoma antigens (SCCA-1 and 2 (7, 8)), maspin (9), proteinase inhibitor 6 (PI-6 (10–12)), proteinase inhibitor 8 (PI-8 (13)), proteinase inhibitor 9 (PI-9 (13, 14)), and proteinase inhibitor 10 (PI-10 (15)).

Comparisons of gene localization and organization suggest that the ovalbumin serpins can be subdivided into two groups exemplified by PI-6 and PAI-2. The PAI-2, SCCA-1, SCCA-2, and maspin genes are on human chromosome 18 (8), whereas the PI-6, PI-9, and MNEI genes are on human chromosome 6 (16–19). This pattern extends to the mouse where the PAI-2 gene is on chromosome 1 (20) and the gene encoding the PI-6 homolog, SPI3, is on chromosome 13 (21). Although otherwise identical in structure, the PI-6 gene lacks an intron present in PAI-2, SCCA-1, and SCCA-2 (8, 21). By amino acid sequence, PI-8 and PI-9 are more like PI-6 (13); whereas PI-10 resembles PAI-2 (15).

The physiological roles of most ovalbumin serpins are unknown but MNEI, maspin, PAI-2, and SCCA may function in tumorigenesis and inflammation. MNEI has been postulated to control serine proteinases found at inflammatory sites to prevent damage to surrounding tissue (22), and maspin is a can-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) U96700–U96709.

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1 The abbreviations used are: PAI-2, plasminogen activator inhibitor 2; graB, granzyme B; MNEI, monocyte/neutrophil elastase inhibitor; PI-6, proteinase inhibitor 6; PI-8, proteinase inhibitor 8; PI-9, proteinase inhibitor 9; serpin, serine proteinase inhibitor; SCCA, squamous cell carcinoma antigen; SSPE, standard saline phosphate; PAGE, polyacrylamide gel electrophoresis; RT, reverse transcriptase; PCR, polymerase chain reaction.
didate tumor suppressor (9). Increases in SCCA levels and release are associated with squamous cell carcinoma (7), while PAI-2 expression is increased in monocytes during inflammation (23).

We have recently demonstrated that PI-9 is a potent inhibitor of the cytotoxic lymphocyte granule serine proteinase, granzyme B (graB), and have suggested that PI-9 is produced by cytotoxic lymphocytes and other immune cells to counter misdirected graB released during target cell destruction (14). To investigate the physiological role of PI-9 we have decided to study it in the mouse, which has obvious advantages for studies involving the immune system.

Here we report the identification of 10 new serpin sequences from the mouse arising from a search for a PI-9 homolog. Among these are two that are probably PI-8 homologs and two that closely resemble human PI-9. The remainder represent ovalbumin serpins that have the hallmarks of functional proteinase inhibitors but have no known human counterparts. Eight of these new serpin sequences (including one of the two PI-9 homologs, designated SPI6) were found in activated lymphocytes. SPI6 was characterized further and shown to be a graB inhibitor. These results indicate that rodents have a much larger array of ovalbumin serpins than humans, which probably serves to balance a larger set of proteinases.

**EXPERIMENTAL PROCEDURES**

**General Methods**—General recombinant DNA methods were performed according to Ref. 24. cDNA probes were labeled with [α-32P]dATP (DuPont NEN) using the random hexamer method (Prime-A-Gene System, Promega).

**Cells and Tissues**—The mouse cytotoxic T cell line R8 has been described (25). Mouse allo-immunizations, isolation of spleen and lymph nodes, and culture of isolated spleen and lymph node cells in interleukin-2 containing media were as described (26). The mouse cytotoxic T cell line R8 has been purchased from Sigma.

**Identification of New Serpins**—Degenerate primers PB117 and PB118 designed to conserved sequences flanking the PI-6 and ovalbumin serpin reactive centers were used as described (14). Total RNA was isolated from cells and tissues as described (27). DNA was synthesized from the RNA using Moloney murine leukemia virus reverse transcriptase and oligo(dT) (24), and amplification and cloning of products was performed according to Ref. 24. cDNA probes were labeled with [α-32P]dATP (DuPont NEN) using the random hexamer method (Prime-A-Gene System, Promega).

**RNA Analysis**—Mouse multiple tissue Northern blots containing 2 μg amounts of mRNA from various mouse tissues (CLONTECH) were probed with a cDNA comprising the 3′-untranslated region of the SPI6 cDNA (nucleotides 1035–1819). Hybridization was carried out at 42 °C in 5 × SSPE, 10 × Denhardt’s, 0.1% SDS, and 50% formamide. Membranes were then washed in 0.1 × SSC and 0.1% SDS at 65 °C and exposed to x-ray film. The membrane was subsequently stripped and re-probed under the same conditions with the mBSA cDNA.

**Chromosomal Localization of Spi6**—The chromosomal localization of Spi6 was mapped by analysis of the Jackson Laboratory (C57BL/ B6JEi) and (C57BL/ B6Je) and Erase-a-Base system was used in Southern hybridizations to identify a PAI-2 expression is increased in monocytes during inflammation (23). Approximately 4 × 105 plaques were screened in each case at low stringency using a partial cDNA clone of human PI-9 (14). Membranes were hybridized at 32 °C in 50% formamid, 5 × SSPE, 0.5% SDS, and 5 × Denhardt’s, then washed in 2 × SSPE, 10 × Denhardt’s, 0.1% SDS, and 0.1% SDS at 45 °C. Positive plaques were purified and the EcoRI inserts were subcloned into the EcoRI site of pUC118 or pBluescript II (Strategene). Both strands of each cDNA were sequenced using the presence of the hinge region motif (Gly-Thr-Glu-Ala-Ala-Ala-Ala(Thr/ Ser)) (2) and a conserved Phe-Cys-Ala-Asp sequence not encoded by the primers.

**cDNA Isolation and Sequencing**—The SPI6 cDNA clone was isolated from a mouse day 15 embryo library in Agt10 (CLONTECH). The mBSA2 and mB171 clones were isolated from a mouse bone marrow library in ggt10 (CLONTECH). Approximately 4 × 105 plaques were screened in each case at low stringency using a partial cDNA clone of human PI-9 (14). Membranes were hybridized at 32 °C in 50% formamid, 5 × SSPE, 0.5% SDS, and 5 × Denhardt’s, then washed in 2 × SSC and 0.1% SDS at 45 °C. Positive plaques were purified and the EcoRI inserts were subcloned into the EcoRI site of pUC118 or pBluescript II KS+ (Strategene). Both strands of each cDNA were sequenced using the Sequenase II system (Amersham) either on overlapping clones generated by directed exonuclease III deletion (Erase-a-Base system, Promega), or by an oligonucleotide walk strategy. Sequences were assembled and analyzed using the DNASSIS programs (Hitachi).

**Preparation of SPI6 Protein for Determination of Complex Forming Activity**—The SPI6 cDNA was subcloned into the EcoRI site of pSVTFI (28) so that the 5′ end of the clone was closest to the T7 promoter. Purified plasmid DNA was linearized with Sall prior to transcription/translation. For control production of human PI-6, pSVFETP/I (11) was linearized with Sall. Labeled protein was produced in a coupled TNT™ Wheat Germ Extract system (Promega) using T7 polymerase and incorporating [35S]methionine (DuPont NEN). Translation products were analyzed by SDS-PAGE (29) or native PAGE (30) followed by fluorography (Amerham). For analysis of complex formation, 2 μl of the translation products were incubated at 37 °C for 30 min with 2 ng of the appropriate proteinase. Purification of active graB was as described (31). Thrombin was purified as described (32), chymotrypsin, trypsin, leukocyte elastase, pancreatic elastase, and cathepsin G were purchased from Sigma.

**Production of Recombinant SPI6**—The SPI6 cDNA was modified by PCR to incorporate a hexa-histidine tag immediately after the initiating methionine. The 1.8-kilobase SPI6 cDNA in Agt10 was used as a template in a PCR primed by 5’-TCTGCCCACATGACATCATCATCATCAT AATTACTCTCTGCTCTGAGAAAATTG-3’ (sense) and 5’-TTATG GAGAGAAGACGCACCA-3’ (antisense). Amplification, cloning, verification, and expression of the modified SPI6 cDNA in Pichia pastoris was as described previously for PI-6 and PI-9 (14, 33).

**SPI6 and GraB Kinetics**—For stoichiometric determinations, 10 pmol of graB was incubated with different concentrations of SPI6 at 37 °C in 20 μl Hepes pH 7.4, 100 mM NaCl, 0.05% (w/v) Nonidet-P40 (34). Residual enzyme activity was determined after 15 min by a two-stage assay using Boc-Ala-Ala-Asp-benzyl and 5,5-dithiobis(nitrobenzic acid) (35). The rate of inhibition of graB by SPI6 was detected by incubating equimolar enzyme and inhibitor at 37 °C, and determining residual activity periodically (33, 36). The second-order rate constant was calculated as described (33).

**Site-directed Mutagenesis**—The SPI6 cDNA cloned in the P. pastoris expression vector pHiL2 was mutated using the Transformer™ kit (CLONTECH). The selection primer 5’-CGGTGACCATGCGACCT TCAAC-3’ removes a unique XbaI in pHIL2. The mutagenic primer 5′-GCCATCATAGCATTTTGCC-3’ substitutes 33Glu in the sequence with Ala. Following verification by DNA sequencing, the mutated SPI6 cDNA was subcloned from pHIL2 to pSVTFI for use in the coupled transcription/translation system.

**RNA Analysis**—Mouse multiple tissue Northern blots containing 2 μg amounts of mRNA from various mouse tissues (CLONTECH) were probed with a cDNA comprising the 3′-untranslated region of the SPI6 cDNA (nucleotides 1035–1819). Hybridization was carried out at 42 °C in 5 × SSPE, 10 × Denhardt’s, 0.1% SDS, and 50% formamide. Membranes were then washed in 0.1 × SSC and 0.1% SDS at 65 °C and exposed to x-ray film. The membrane was subsequently stripped and re-probed under the same conditions with the mBSA cDNA.

**Identification of GraB Binding Activity and Multiple Serpin Sequences in Mouse Immune Cells**—We have previously reported that PI-9 is an inhibitor of graB, and that it is predominantly expressed in immune cells (14). To assess whether a comparable inhibitor exists in mouse cells, we incubated a cytosolic extract from R8 cells with purified human graB. R8 cells are an interleukin-2-dependent mouse cytotoxic lymphocyte line that produce granules and are reminiscent of natural killer cells (25). In many respects they resemble the human YT line which we have shown produces large quantities of graB and PI-9 (14).

As shown in Fig. 1, incubation of R8 extracts with graB led to the formation of a 67-kDa complex detected by SDS-PAGE and immunoblotting with an anti-graB monoclonal antibody. This resembled the SDS-stable complex formed between graB and PI-9, which is characteristic of serine proteinase-serpin interactions (2). In addition, immunoblotting with anti-PI-9 antibodies weakly detected a 42-kDa protein in R8 extracts (data not shown). From these experiments we concluded that a mouse graB inhibitor and homolog of PI-9 is present in R8 cells.

In a parallel approach we used RT-PCR to identify ovalbumin serpin sequences present in mouse immune tissue. Given its presence in activated lymphocytes (14), it is conceivable that PI-9 or its homologs are primarily produced during an immune response. To allow for this, we took cells from the popliteal lymph nodes and spleens of allo-immunized mice. Some of
these lymph node and spleen cells were then cultured for 1–2 weeks in the presence of interleukin-2, which selectively activates and promotes the proliferation of cytotoxic lymphocytes (in vitro stimulated). R8 cells and a long-term (>6 months) culture of mouse spleen cells passed through various levels of interleukin-2 were also examined. The latter cells (designated NK) exhibit natural killer-like, non-MHC restricted cytotoxic function.

RNA was prepared from each of these sources and used as a template for cDNA production and PCR. The PCR primers were degenerate oligonucleotides designed to ovumblin serpin sequences that have been previously used to identify PI-9 (14). These amplify the serpin reactive center loop and candidate sequences are identified by conserved residues inside the primer-binding sites. In our hands this primer pair has amplified PAI-2, MNEI, PI-6, PI-8, PI-9, and heparin cofactor II from various human tissues.

As shown in Fig. 2, RT-PCR of the mouse cells and tissue identified nine serpin sequences (SPI3, SPI6, mNK10, mAT2, mR86, mNK9, mNK13, mNK21, and mNK26). One of these (SPI3) is the previously described mouse homolog of PI-6 (21). Reactive center bonds (P1-P1 bond) unique feature of PI-9 (14) and is crucial for graB inhibition, 4 with the other serpins suggested that a Glu would be at, or close to, the P3-P1 bond. Since (i) a Glu at this position is a unique feature of PI-9 (14) and is crucial for graB inhibition, (ii) SPI6 sequences arose most frequently in R8 cells which also contain a graB-binding function, and (iii) the other serpin (SPI3) observed in R8 cells does not interact with graB, we hypothesized that SPI6 is a functional homolog of PI-9.

**Isolation and Sequencing of SPI6 cDNAs—**To isolate a complete SPI6 cDNA, mouse bone marrow and day 15 embryo cDNA libraries were hybridized at low stringency to a partial prototype serpin sequence, alpha-antiproteinase (1). Lower panel shows the mouse serpin sequences identified in this study. SPI3 is the mouse homolog of PI-6 (21). Reactive center bonds (P1-P1) are indicated by the vertical arrow. Dashes indicate gaps introduced for optimum sequence alignment. The sequences used for the design of the degenerate PCR primers are indicated by the horizontal arrows above the PAI-9 sequence.

Three clones were isolated from the mouse embryo library. Two corresponded to the characterized serpins SPI3 and mouse alpha-antitrypsin, while the third clone contained a 1.8-kilobase insert encoding SPI6. (The designation serine proteinase inhibitor 6 (SPI6; gene Spil6) has been allocated by the International Committee on Standardized Nomenclature for Mice.)

Sequencing of the SPI6 cDNA predicted a protein of 374 amino acids (Fig. 3A). The coding region is flanked by a short 5′-untranslated region containing two termination codons (one in frame with the first methionine), and is followed by a 653-

**Fig. 1. Identification of a graB binding activity in mouse cells.** Cytosolic extracts were prepared from R8 cells and incubated in the absence or presence of 10 ng of purified human graB. Samples were boiled and reduced prior to electrophoresis on a 12% SDS-polyacrylamide gel, then immunoblotted with a graB monoclonal antibody. The arrow shows the position of the SDS-resistant complex containing graB.

**Fig. 2. New mouse serpin sequences identified by cDNA library screening and RT-PCR. Upper panel** shows the inhibitory region reactive center loop sequences of the previously characterized human ovumblin serpins PAI-2 (5), SCCA-1 (7), SCCA-2 (8), maspin (9), PI-6 (11), PI-8 (13), PI-9 (13, 14), PI-10 (15), and MNEI (6), and the prototype serpin sequence, alpha-antiproteinase (1). Lower panel shows the mouse serpin sequences identified in this study. SPI3 is the mouse homolog of PI-6 (21). Reactive center bonds (P1-P1) are indicated by the vertical arrow. Dashes indicate gaps introduced for optimum sequence alignment. The sequences used for the design of the degenerate PCR primers are indicated by the horizontal arrows above the PAI-9 sequence.
base pair 3'-untranslated region which includes a polyadenylation signal at nucleotides 1684. However, the cDNA extends for a further 135 base pairs and has no poly(A) tail, suggesting that Spi6 transcription can continue past this first polyadenylation signal. Transcriptional termination and polyadenylation might then be directed by another signal not represented in this clone. Two polyadenylation signals would allow transcriptional termination to occur at either of two points, and may explain the presence of two Spi6 transcripts detected by Northern blotting (see Fig. 5A).

Sequencing of the mBM2A partial clone revealed a serpin highly related (85%) to Spi6 (Fig. 3B). Compared with other serpins, Spi6 and mBM2A are most like PI-9 (68 and 65% identity, respectively). Spi6 and mBM2A show greatest identity to the PI-6-like serpins suggesting they are members of this ovalbumin serpin subgroup. For example, Spi6 shows 55% identity with SPI3, 48% with MNEI, and 55% with PI-8 (Fig. 4), consistent with its role as an inhibitor of a neutral proteinase like PI-9 (3). The reaction stoichiometry is usually equimolar and initial formation of a Michaelis complex is followed by the formation of a kinetically stable (locked) tetrahedral complex (2, 3). To examine the interaction of Spi6 and graB in more detail, we first established the stoichiometry of the reaction by titrating a fixed amount of graB against varying amounts of inhibitor and measuring residual proteolytic activity (data not shown). The 1:1 ratio observed is typical of a serine proteinase-serpin interaction (2). The association rate constant (kₐ) for complex formation was calculated as 8 × 10⁴ M⁻¹ s⁻¹. This is just outside the range for physiologically important serpin-proteinase interactions (2, 3), and is somewhat lower than the constant for the graB-PI-9 interaction (1.7 × 10⁶ M⁻¹ s⁻¹ (14)). This poorer than expected inhibition may simply reflect the different species origin of the serpin and proteinase (see "Discussion").

Expression of Spi6 and Interaction with Serine Proteinases—[³⁵S]Methionine-labeled Spi6 was produced in vitro using a coupled transcription/translation system. The translation products were analyzed by SDS-PAGE and fluorography. As predicted from the sequence, a protein of approximately 42 kDa was produced from the Spi6 cDNA (Fig. 5). Labeled Spi6 was then tested for complex formation with graB and a number of other serine proteinases. A feature of serpin-serine proteinase interactions is the formation of a stable complex that is not dissociated by SDS (2). Addition of labeled Spi6 to purified graB resulted in the formation of a 67-kDa complex that was apparent following reduction, boiling, and electrophoresis in SDS (Fig. 5). Human PI-9, which forms an SDS-stable complex with graB (14), was produced in the same system and incubated with graB. As expected, a 67-kDa graB-PI-9 complex was observed (Fig. 5).

Spi6 did not complex with any of the following proteinases: trypsin, chymotrypsin, leukocyte (neutrophil) elastase, pancreatic elastase, thrombin, or cathepsin G (data not shown). Similar results were obtained by native PAGE (data not shown). These experiments suggest that Spi6 is a likely mouse homolog of PI-9.

Inhibition of Human GraB by Spi6—Hexa-histidine tagged recombinant Spi6 was produced in a yeast expression system and purified by nickel affinity chromatography. As predicted from the cDNA sequence, the purified recombinant protein had a molecular mass of 42 kDa (not shown).

Complex formation between a serpin and proteinase follows second-order kinetics, and association rate constants of 10⁶ to 10⁸ M⁻¹ s⁻¹ represent physiologically significant interactions (3). The reaction stoichiometry is usually equimolar and initial formation of a Michaelis complex is followed by the formation of a kinetically stable (locked) tetrahedral complex (2, 3). To examine the interaction of Spi6 and graB in more detail, we first established the stoichiometry of the reaction by titrating a fixed amount of graB against varying amounts of inhibitor and measuring residual proteolytic activity (data not shown). The 1:1 ratio observed is typical of a serine proteinase-serpin interaction (2). The association rate constant (kₐ) for complex formation was calculated as 8 ± 0.8 × 10⁴ M⁻¹ s⁻¹. This is just outside the range for physiologically important serpin-proteinase interactions (2, 3), and is somewhat lower than the constant for the graB-PI-9 interaction (1.7 × 10⁶ M⁻¹ s⁻¹ (14)). This poorer than expected inhibition may simply reflect the different species origin of the serpin and proteinase (see "Discussion").

Role of Glu³⁷ in Spi6 Inhibitory Function—A unique feature of PI-9 is the presence of an acidic residue (Glu) at the P₁ position, which is consistent with its role as an inhibitor of a proteinase cleaving after acidic residues (Asp or Glu). Substitution of the P₁ Glu in PI-9 dramatically lowers inhibitory activity against graB. Because Spi6 and mBM2A have shorter reactive center loops than PI-9 and other ovalbumin serpins, it is difficult to identify the likely P₁ residues by sequence alignment alone. However, both Spi6 and mBM2A have only one acidic residue (Glu) in their reactive center loops, and it is likely that this is important for inhibition of graB. To test this we used site-directed mutagenesis to produce a derivative of

![Table I](image)

| Sequence  | R8 cells | NK cells | Spleen | Popliteal | Spleen in vitro | Popliteal in vitro |
|-----------|----------|----------|--------|-----------|----------------|--------------------|
| SPI3      | 26/45    | 23/35    | 49/69  | 15/35     | 4/23           | 32/36              |
| SPI6      | 16/45    | 4/35     | 3/69   | 3/23      | 2/23           | 2/36               |
| mBM2A     | 0/45     | 0/35     | 0/69   | 0/35      | 0/23           | 0/36               |
| mBM17     | 0/45     | 0/35     | 0/69   | 0/35      | 0/23           | 0/36               |
| mNK10     | 0/45     | 1/35     | 3/69   | 10/35     | 3/23           | 0/36               |
| mA72      | 0/45     | 0/35     | 0/69   | 0/35      | 2/23           | 0/36               |
| mR88      | 3/45     | 0/35     | 2/69   | 4/35      | 1/23           | 1/36               |
| mNK9      | 0/45     | 2/35     | 1/69   | 2/35      | 2/23           | 0/36               |
| mNK13     | 0/45     | 2/35     | 11/69  | 1/35      | 5/23           | 1/36               |
| mNK21     | 0/45     | 2/35     | 0/69   | 1/35      | 3/23           | 0/36               |
| mNK26     | 0/45     | 1/35     | 0/69   | 0/35      | 0/23           | 0/36               |

*RT-PCR products were cloned and analyzed by DNA sequencing and/or hybridization to specific oligonucleotides. Sequences are shown in Fig. 2. Spleen and popliteal in vitro samples indicate cells taken from allo-immunized mice and cultured for 3–5 days in the presence of interleukin-2 prior to preparation of RNA.
SPI6 in which Glu337 has been substituted by Ala. This derivative was produced in the coupled transcription/translation system and in the yeast expression system. As shown in Fig. 5, labeled mutant protein did not form a detectable complex with graB on SDS-PAGE. In addition, although we were able to produce and purify equivalent amounts of the mutant from the yeast system (compared with SPI6), we were unable to demonstrate any graB inhibitory activity (data not shown). We conclude that Glu337 is crucial for the inhibitory activity of SPI6, and that it may represent the P 1 residue.

Tissue Distribution of SPI6 and mBM2A mRNA—To determine the tissue distribution of SPI6 and mBM2A, a membrane containing mRNA from mouse brain, heart, kidney, liver, lung, skeletal muscle, spleen, and testis was sequentially hybridized to 32P-labeled SPI6 and mBM2A cDNA probes. With an SPI6 3′-untranslated region probe, two transcripts of approximately 2.4 and 3.6 kilobases were detected in most tissues, but predominantly in heart, lung, spleen, and kidney. The highest levels were in lung and spleen (Fig. 6). The nature of the two transcripts is unknown but they may arise by differential splicing or (as discussed above) differential transcriptional termination.

When the same membrane was stripped and hybridized to the mBM2A cDNA, the most prominent signal was obtained in testis (Fig. 6). Here a single 1.7-kilobase transcript was observed, that was not detected in other tissues. Fainter bands of identical size, distribution, and relative intensities as those seen with the SPI6 probe were also observed. These are likely to represent cross-hybridization of the mBM2A probe with SPI6 transcripts. We conclude that while mBM2A expression is essentially restricted to testis, SPI6 expression is somewhat broader.

Chromosomal Localization of Spi6—The PI-6, human MNEI, and PI-9 genes co-localize on human chromosome 6p25 (17, 18, 20, 40), while the PAI-2, SCCA, and maspin genes are clustered on human chromosome 18q21 (8). If SPI6 is a new member of the PI-6 family it is likely that the gene will localize to the same region on chromosome 13 as the gene encoding the mouse PI-6 homologue, SPI3.

The chromosomal localization of Spi6 was determined by hybridization of a partial SPI6 cDNA to a panel of DNAs from interspecific backcross mice (37). The SPI6 fragment (798–1819 base pairs) detected a PvuII polymorphism between M. musculus (C57BL/BJEi) and M. spretus (SPRET/Ei) and was then hybridized to PvuII digested genomic DNA from each of the 94 animals derived from a (M. musculus x M. spretus)F1 x M. spretus backcross. After scoring each sample for the presence or absence of the M. musculus fragment, Spi6 was localized to mouse chromosome 13 in the same region as mouse Spi3. Spi6 and Spi3 could not be separated by this analysis, indicating a distance between them of 0–3.8 centimorgan. Data in Table II show the recombination frequencies for the nearest linkage markers for Spi6. Spi6 is localized approximately 20 centimorgans from the centromere of chromosome 13 between the placental lactogen 1 gene (3.2 centimorgans proximal) and the motif-primed PCR marker D13Bir9 (1.1 centimorgans distal).

DISCUSSION

In this study we report the identification of 10 previously unknown serpin sequences in the mouse. Most of the new sequences were identified by RT-PCR using primers specifically designed to amplify ovalbumin serpin inhibitory regions, and sequence analysis shows that each new serpin has a Phe-Cys-Ala-Asp motif in the distal portion of this region that is present in most human ovalbumin serpins but not in other members of the serpin superfamily. Thus these new mouse serpins are distinct from the other known mouse serpins.

FIG. 3. Sequences of SPI6 and mBM2A. A, cDNA and polypeptide sequence of SPI6. B, amino acid sequence comparison of SPI6 and the partial sequence mBM2A. The inhibitory region containing the reactive center loop (2, 39) is boxed.

SPI6 in which Glu337 has been substituted by Ala. This derivative was produced in the coupled transcription/translation system and in the yeast expression system. As shown in Fig. 5, labeled mutant protein did not form a detectable complex with graB on SDS-PAGE. In addition, although we were able to produce and purify equivalent amounts of the mutant from the yeast system (compared with SPI6), we were unable to demonstrate any graB inhibitory activity (data not shown). We conclude that Glu337 is crucial for the inhibitory activity of SPI6, and that it may represent the P 1 residue.

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When the same membrane was stripped and hybridized to the mBM2A cDNA, the most prominent signal was obtained in testis (Fig. 6). Here a single 1.7-kilobase transcript was observed, that was not detected in other tissues. Fainter bands of identical size, distribution, and relative intensities as those seen with the SPI6 probe were also observed. These are likely to represent cross-hybridization of the mBM2A probe with SPI6 transcripts. We conclude that while mBM2A expression is essentially restricted to testis, SPI6 expression is somewhat broader.

Chromosomal Localization of Spi6—The PI-6, human MNEI, and PI-9 genes co-localize on human chromosome 6p25 (17, 18, 20, 40), while the PAI-2, SCCA, and maspin genes are clustered on human chromosome 18q21 (8). If SPI6 is a new member of the PI-6 family it is likely that the gene will localize to the same region on chromosome 13 as the gene encoding the mouse PI-6 homologue, SPI3.

The chromosomal localization of Spi6 was determined by hybridization of a partial SPI6 cDNA to a panel of DNAs from interspecific backcross mice (37). The SPI6 fragment (798–1819 base pairs) detected a PvuII polymorphism between M. musculus (C57BL/BJEi) and M. spretus (SPRET/Ei) and was then hybridized to PvuII digested genomic DNA from each of the 94 animals derived from a (M. musculus x M. spretus)F1 x M. spretus backcross. After scoring each sample for the presence or absence of the M. musculus fragment, Spi6 was localized to mouse chromosome 13 in the same region as mouse Spi3. Spi6 and Spi3 could not be separated by this analysis, indicating a distance between them of 0–3.8 centimorgan. Data in Table II show the recombination frequencies for the nearest linkage markers for Spi6. Spi6 is localized approximately 20 centimorgans from the centromere of chromosome 13 between the placental lactogen 1 gene (3.2 centimorgans proximal) and the motif-primed PCR marker D13Bir9 (1.1 centimorgans distal).

DISCUSSION

In this study we report the identification of 10 previously unknown serpin sequences in the mouse. Most of the new sequences were identified by RT-PCR using primers specifically designed to amplify ovalbumin serpin inhibitory regions, and sequence analysis shows that each new serpin has a Phe-Cys-Ala-Asp motif in the distal portion of this region that is present in most human ovalbumin serpins but not in other members of the serpin superfamily. Thus these new mouse serpins are distinct from the other known mouse serpins.
serpins are probably members of the ovalbumin serpin sub-
group. Based on the presence of consensus proximal hinge 
motifs each of these serpins is likely to be a proteinase inhibi-
tor, but in the absence of functional data possible targets of 
these serpins cannot easily be predicted. It should be noted, 
however, that recent studies have shown that serpins are ca-
pable of inhibiting more than one class of proteinase (34, 41). 
Hence the cognate proteinases of these mouse serpins may 
include serine proteinases and cysteine proteinases (caspases 
or papain-like cathepsins).

Even though our search for mouse ovalbumin serpins was 
largely restricted to activated lymphocytes, the number of new 
sequences identified suggests that the ovalbumin serpin family 
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FIG. 4. Comparison of SPI6 with the PI-16 ovalbumin serpins. The numbering, conserved amino acids and structural elements indicated by 
lines above the sequence are based on the structure of α1-antiproteinase (1). The signal sequence removed to generate mature α1-antiproteinase 
is boxed. Reactive center bonds (P1-P1') are indicated by the vertical arrow. Dashes indicate gaps introduced for optimal sequence alignment. 
Sequences indicated are α1-antiproteinase (1), SPI3 (21), PI-6 (11), PI-8 (13), PI-9 (13, 14), and MNEI (6).

FIG. 5. Interaction of SPI6 and an SPI6 inhibitory region mu-
ant with grαB. 35S-Labeled SPI6, PI-9, mutated SPI6 (Glu337 to Ala), 
and mutated PI-9 (Glu340 to Ala) were produced in a coupled transcrip-
tion/translation system. Products were incubated with or without 2 ng 
of human grαB for 15 min at 37 °C, reduced, and complex formation was 
analyzed following 12% SDS-PAGE and fluorography.

FIG. 6. Tissue distribution of SPI6 and mBMB2A mRNA. Upper panel, a membrane containing 2-μg samples of mRNA from a variety of 
mouse tissues was hybridized to a 32P-labeled SPI6 probe consisting 
mainly of the 3' untranslated region (from nucleotide 1035). The final 
wash was in 0.1× SSC at 60 °C for 30 min. Lower panel, the membrane 
was stripped and hybridized to a 32P-labeled mBMB2A probe consisting 
of coding and 3' untranslated sequences. Washing conditions and ex-
posure times were as exactly the same as the first hybridization.

in the mouse is much larger than its human counterpart. 
The existence of many more serpins in the mouse has already 
been suggested by the description of two gene clusters on mouse 
chromosome 12 that have only single human gene equivalents 
(42, 43). These clusters have almost certainly arisen by multi-

ple gene duplications and encode serpins resembling α1-antitrypsin (Spi1) or α1-antichymotrypsin (Spi2). It is likely that, although closely related, individual members of each cluster have different inhibitory capacities. The family of mouse ovalbumin serpins identified here have at least three human counterparts (PI-6, PI-8, and PI-9) and almost certainly also target a diverse range of proteinases. The fact that Spi6 maps to the same chromosomal region as Spi3 suggests that a serpin gene cluster comprising some of the mouse ovalbumin serpins is present on chromosome 13, and that SPI6 is a member of the PI-6 ovalbumin serpin subfamily and not the PAI-2 group. This is supported by sequence comparisons which show that SPI6 more closely resembles mouse PI-6 (SPI3, 55%) than mouse PAI-2 (40%). (Fig. 4 shows a comparison of SPI6 with the other members of the PI-6 group.) It remains to be seen whether Spi6 resembles Spi3 in gene organization and lacks an intron present in PAI-2, SCCA-1, SCCA-2, and ovalbumin (21).

The larger array of inhibitory serpins in the mouse suggests that rodents have many more proteinases that must be regulated. This is illustrated by a comparison of the granule proteinase complements of cytotoxic lymphocytes in humans and rodents. In humans there are five granule serine proteinases whereas in rodents there are at least nine (44). It is conceivable that some of the new serpins we have identified that lack obvious human counterparts are involved in regulating granule serine proteinases specific to the mouse. Since most of these new serpins are likely to be lymphocyte products it is probable that other mouse leukocytes have a distinct ovalbumin serpin complement, perhaps with novel members not identified here. For example, monocytes and neutrophils should have one or more counterparts of PAI-2 and MNEI. At present only one mouse homolog of PAI-2 is known (20). Due to the primer design, we may not have amplified non-inhibitory serpin sequences so it is also conceivable that non-inhibitory mouse ovalbumin serpins await discovery.

An interesting observation arising from this work is the apparent existence of multiple mouse homologs of at least two human ovalbumin serpins. Specifically, the clones mBM17 and mNK10 are probably homologs of human PI-8, while the clones mBM2A and SPI6 are homologs of human PI-9. It is possible that the different homologs play distinct roles in the mouse. For example, SPI6 and mBM2A may both regulate graB, but do so in separate contexts. This is supported by the fact that mBM2A and SPI6 have a different tissue distribution. Alternatively, these two serpins may regulate related but distinct proteinases within the mouse. Similarly, mBM17 and mNK10 probably have different roles since a different tissue distribution is implied by the presence of mNK10, and absence of mBM17, in lymphocytes.

On the basis of its properties and distribution, we have proposed that PI-9 functions to protect cytotoxic lymphocytes and other immune system components, such as antigen-presenting cells, from mis-directed graB (14). It is most likely that SPI6 is the true PI-9 homolog because (i) it has over 68% sequence identity with PI-9; (ii) it is also a graB inhibitor; (iii) inhibition depends on a glutamic acid residue in the reactive loop; and (iv) like PI-9 it is present in lymphocytes and spleen cells. However, SPI6 shows a broader tissue distribution than PI-9, being present in heart, lung, and kidney. At present we do not know whether this indicates an additional function for a graB inhibitor in the mouse, or whether it simply represents a different distribution of antigen-presenting or cytotoxic (CD8+ and CD56+) cells in mouse tissues.

Although we have been unable to do functional studies with mBM2A, its expression pattern (predominant in testis, absent in lymphocytes and spleen cells) rules it out as a direct homolog of PI-9. If mBM2A is a graB inhibitor, its presence in mouse testis may be related to the immune-privileged status of certain cells within the testis. It has been shown recently that Sertoli cells in the mouse testis express Fas ligand, and can induce apoptosis of cytotoxic lymphocytes that come in contact with them (for review, see Ref. 45). It may be that mouse Sertoli cells also produce a graB inhibitor (mBM2A) to protect against the granule-mediated arm of the CL killing machinery. Alternatively, mBM2A may regulate a different proteinase to graB. Recent evidence suggests that there is more than one serine aspase in rodents.

One problem with the proposal that SPI6 and PI-9 are homologs is the relative inefficiency with which SPI6 inhibits human graB, but this may be a consequence of the lower than expected sequence conservation (39%) in the SPI6 and PI-9 inhibitory loops. Specifically, the SPI6 loop is two amino acids shorter than PI-9, there is an aromatic amino acid near the P1,P2,P3 residues in SPI6 but not in PI-9, and the putative P1 glutamic acid residue is much closer to the proximal hinge in SPI6 than in PI-9. Obviously these differences may reflect structural variations in the active sites of mouse and human graB, and in their substrate specificity. Formal resolution awaits the availability of practical quantities of purified mouse graB to test with SPI6 and PI-9. We expect that SPI6 will inhibit mouse graB more efficiently than human graB.

In conclusion, we have provided evidence for a large family of ovalbumin serpins in mouse lymphocytes and marrow, and the existence of multiple homologs of two human intracellular serpins. The distribution and properties of these new serpins suggests that rodents possess additional proteolytic systems that contribute to immune function. This might reflect differing evolutionary pressures placed on rodent and human immune systems, and suggests that caution should be exercised in extrapolating results from rodent models to humans.

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### TABLE II

Mapping of the mouse SPI6 gene to chromosome 13 in a (M. musculus x M. spretus)F1 x M. spretus backcross typing panel

| Interval | Recombinant/total | Recombination frequency (×100) ± S.E.M. | 95% confidence limits (lower-upper) |
|----------|------------------|----------------------------------------|-----------------------------------|
| Gpd11-P1 | 1/94             | 1.96 ± 1.06                            | 0.9–5.8 centimorgans              |
| P1-Spi3  | 3/94             | 3.23 ± 1.83                            | 0.7–9.1 centimorgans              |
| Spi3-Spi6| 0/94             | 0                                      | 0–4.0 centimorgans                |
| Spi6-D13Bir9 | 0/94   | 1.06 ± 1.06                            | 0.0–6.0 centimorgans              |
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