

# A new intracellular serine protease inhibitor expressed in the rat pituitary gland complexes with granzyme B

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**Abstract** We have cloned a novel serpin (raPIT5a) from a rat pituitary cDNA library which is structurally related to members of the ovalbumin subfamily of serine protease inhibitors. This new cDNA encodes a 374-amino acid protein, designated raPIT5a. raPIT5a was expressed in specific cells in the intermediate and anterior lobes of the pituitary. Recombinant raPIT5a was not secreted suggesting raPIT5a functions to inhibit intracellular proteases. Recombinant raPIT5a formed an SDS-stable complex with human granzyme B, a serine protease which induces apoptosis by activating members of the caspase enzyme family. These data suggest raPIT5a may have a role in regulating granzyme B or related enzymes and apoptosis in the pituitary gland.

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**Key words:** Inhibitor; Pituitary; Serpin; Serine protease; Granzyme B

## 1. Introduction

Serine protease inhibitors or serpins are a large family of proteins which have been identified from sources as diverse as viruses and humans. The serpin family includes most of the protease inhibitors found in blood as well as many other proteins with unrelated or unknown functions. Members include  $\alpha_1$ -proteinase inhibitor (also called  $\alpha_1$ -antitrypsin), antithrombin, plasminogen activator inhibitor-1 and the cowpox viral serpin, cytokine response modifier A [1–4]. Serpins play a critical role in the control of proteolysis.  $\alpha_1$ -Proteinase inhibitor is the principal inhibitor of neutrophil elastase [5]. Antithrombin is an inhibitor of most of the serine proteases involved in the blood coagulation cascade [6]. Plasminogen activator inhibitor-1 inhibits the plasminogen activators t-PA and u-PA [7–9]. Cytokine response modifier A inhibits interleukin converting enzyme, a cysteine protease identified as being important for programmed cell death [10].

Endocrine tissues express a range of serine proteases including enzymes involved in the biosynthesis of biologically active peptides [11], the regulation of cellular responses to biologi-

cally active peptides [12] and ovulation [13]. These proteases are likely to be regulated by specific inhibitors [14]. The evident importance of serpins in endocrine physiology led us to initiate a new research project to identify novel serine protease inhibitors in the pituitary using a polymerase chain reaction-based screening strategy. In this study we report the cloning, sequencing and expression of a new serpin which is related to the recently identified ovalbumin subfamily of serpins.

## 2. Materials and methods

### 2.1. Cloning and sequence analysis of raPIT5a

Two degenerate oligonucleotide primers were synthesised based on conserved sequences that bracket the reactive centre residues and other conserved regions within the carboxy-terminus of serpins. These primers (sense, 5'-G(CA)(CA)(GA)GTGCCCATGATG-3'; antisense, 5'-A(ACGT)(CA)A(GT)GAA(TA)GG(CG)(CT)(GT)GT-3') were used to amplify a ~400-bp fragment from rat pituitary first strand cDNA. The amplified DNA was subcloned into M13mp18 or M13mp19, phage from individual recombinants purified and the insert DNA amplified using M13 forward and reverse sequencing primers. Amplified DNA was restricted with *Hinf*I and *Hae*III. DNAs that showed unique fragmentation patterns with either enzyme were further characterised by DNA sequence analysis using an ABI Model 370 DNA sequencer and a unique serpin sequence, named raPIT5a, identified. Using this sequence as a probe, a ~3.3-kb raPIT5a clone was isolated from a rat pituitary cDNA library and sequenced in both directions.

### 2.2. In situ hybridisation histochemistry

Two RNA probes were prepared to raPIT5a sequences 235–697 and 714–1112 using T7 and T3 RNA polymerase (Ambion, Texas, USA) and uridine 5'-( $\alpha$ -thio)triphosphate (Dupont NEN, Massachusetts, USA). Each probe was generated using the polymerase chain reaction and included T3 and T7 primer sequences at the 5' termini to allow the preparation of sense and antisense probes. Details of the in situ hybridisation histochemistry methodology can be found at <http://intra.nimh.nih.gov/lcmr/snge/>. Tissue sections were hybridised with sense and antisense riboprobes encoding either the 5' or 3' probe sequences. Sections were opposed to Biomax MS film and then dipped in NTB3 nuclear track emulsion (Kodak). Sections were exposed to emulsion for 20 days at 4°C before development with Kodak Dektol at 15°C and counterstaining with cresyl violet. All animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

### 2.3. Expression and analysis of recombinant raPIT5a

A pGem-raPIT5a construct containing a 2.9-kb raPIT5a *Bam*HI fragment was used to direct expression of recombinant raPIT5a in BSC-40 cells using the vaccinia virus/T7 RNA polymerase hybrid system. A 10-cm dish was seeded with  $4 \times 10^6$  BSC-40 cells and infected the following day for 30 min with  $4.5 \times 10^6$  PFU vTF7-3 (recombinant virus expressing the T7 RNA polymerase gene). The cells were then incubated overnight with a pGem-raPIT5a/Lipofectamine suspension (Life Technologies) and after incubation for a further 2 days, cells and media analysed for raPIT5a expression.

To determine whether recombinant raPIT5a was secreted or re-

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**Abbreviations:** Serpin, serine protease inhibitor; raPIT5a, rat pituitary serine protease inhibitor 5a; PI-8, protease inhibitor-8; PI-9, protease inhibitor-9; PI-6, protease inhibitor-6; SPI6, CrmA, cytokine response modifier A; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; PAI-2, plasminogen activator inhibitor-2

1 TACTGGGAGCATCAAGGCTGCAAAATGCTGGATTGAGAAACCACCAACTGTGACTCTGGATC 12  
 13 ATCTCTGCTAGGCC 72  
 73 ATG AAT ACT CTG TCT GAA GCA AAT GGC ACC TTT GCC ATC CAT CTT 117  
 Met Asn Thr Leu Ser Glu Ala Asn Gly Thr Phe Ala Ile His Leu  
 118 TTG AAG ATG CTA TGT CAA AGC AAC CCT TCA GAA AAT GTA TGT TAC 162  
 Lys Met Leu Cys Gln Ser Asn Pro Ser Glu Asn Val Cys Tyr  
 163 TCT CCT GTG AGC ATC TCC TCT GCT CTA GCT ATG GTC CTC TTG GGG 207  
 Ser Pro Val Ser Ile Ser Ser Ala Leu Ala Met Val Leu Leu Gly  
 208 GCA AAG GGA CAG ACC CAA GTC CAG ATA TCT CAG GCA CTT GGT TTG 252  
 Ala Lys Gly Gln Thr Gln Val Gln Ile Ser Gln Ala Leu Gly Leu  
 253 AAC AAA GAG AAA GAC CTC CAT CAG GGC TTC CAG TTG CTT CTC AGT 297  
 Asn Lys Glu Lys Asp Leu His Gln Leu Leu Leu Leu Ser  
 298 AAC CTG AAC AAG CCA GAG AGA AAA TAC TCC CTT AGA GTG GCC AAC 342  
 Asn Leu Asn Lys Pro Glu Arg Lys Tyr Ser Leu Arg Val Ala Asn  
 343 AGC CTT TTT GCA GAC AAA ACT TGT GAA CTC CTC CCA ACC TAT AAG 387  
 Arg Leu Phe Ala Asp Lys Thr Cys Glu Leu Leu Pro Thr Tyr Lys  
 388 GAG TCC TGT CTT CGA TTC TAT AAT TCA GAG ATG GAG CAG CTC TCC 432  
 Glu Ser Cys Leu Arg Phe Tyr Asn Ser Glu Met Glu Leu Leu Ser  
 433 TTT GCC GAA GCT GCA GAG GAG TCA AGG AAA CAC ATA AAC ACG TGG 477  
 Phe Ala Glu Ala Ala Glu Glu Ser Arg Lys His Ile Asn Thr Trp  
 478 CTC TCC AAA CAG ACT GAA GGC AAA ATT CCT GAG CTG TTG TCA GGT 522  
 Val Ser Lys Gln Thr Glu Gly Lys Ile Pro Glu Leu Leu Ser Gly  
 523 GGT TCA GTT GAT TCA GAG ACC AGG CTG GTT CTC GTC AAT GCC TTA 567  
 Gly Ser Val Asp Ser Glu Thr Arg Leu Val Leu Val Asn Ala Leu  
 568 TAT TTC AAA GGA AGG TGG CAT CAA CCA TTT AAC AAA GAA TAC ACA 612  
 Tyr Phe Lys Gly Arg Trp His Gln Pro Phe Asn Lys Glu Tyr Thr  
 613 CTG GAT ATG CCC TTT AAA ATA AAC AAG AAT GAG AAA CGA CTA GTG 657  
 Val Asp Met Pro Phe Lys Ile Asn Lys Asn Glu Lys Arg Leu Val  
 658 CAG ATG ATG TGT TGT GAG GAC ACA TAT AAC CTT GCC CAT GTG AAG 702  
 Glu Met Met Cys Cys Glu Ser Thr Tyr Asn Leu Ala His Val Lys  
 703 GAG GTG CAG GCG CAA GTG CTC ATG ATG ATG ATG ATG ATG ATG ATG ATG 747  
 Glu Val Gln Ala Gln Val Leu Met Met Pro Tyr Glu Gly Met Glu  
 748 CTG AGC TTC GTG GTC CTG CTC CCA GAT AAT GAC GGG GAC CTC AGC 792  
 Leu Ser Phe Val Val Leu Leu Pro Asp Asn Asp Gly Asp Leu Ser  
 793 AAG GTG GAA AGC AAT CTC ACT TTT GAG AAG TTA ACA GCC TGG ACC 837  
 Lys Val Glu Ser Asn Leu Thr Phe Glu Lys Leu Thr Ala Trp Thr  
 838 AAC CCA GAC TTT ATG AAG AAC ACT AAT GTT GAG GTT TTC CTT CCA 882  
 Asn Pro Asp Phe Met Lys Asn Thr Asn Val Glu Val Phe Leu Pro  
 883 AAA TTT AAA CTG CAA GAG GAT TAT GAC ATG GAG TCT GTG TTT CAG 927  
 Lys Phe Lys Leu Gln Glu Asp Tyr Asp Met Glu Ser Val Phe Gln  
 928 CGC TTG GGG ATA GTG GAT GTC TTC CAA GAG GCC AAG GCT GAC TTA 972  
 Arg Leu Gly Ile Val Asp Val Phe Gln Glu Ala Lys Ala Asp Leu  
 973 TCA GCA ATG TCT CCA GAG AGA AAC CTG TGT GTG TCC AAG ATT GTT 1017  
 Ser Ala Met Ser Pro Glu Arg Asn Leu Cys Val Ser Lys Ile Val  
 1018 CAC AAG AGC GTA GTG GAG GTC AAT GAG GAA GGC ACA GAG GCT GCA 1062  
 His Lys Ser Val Val Glu Val Asn Glu Glu Gly Thr Glu Ala Ala  
 1063 GCA GCC TCC GCT GTC ATA GAA TAC TGC TGT GCT GCT TTT GTC CCA 1107  
 Ala Ala Ser Ala Val Ile Glu Tyr Cys Cys Ala Ala Phe Val Pro  
 1108 ACA TTC TGT GCT GAC CAC CCC TCT CTT TTC TTC ATC AAC CAC AAC 1152  
 Thr Phe Cys Ala Asp His Pro Phe Leu Phe Phe Ile Lys His Asn  
 1153 AAA ACC AAA AGC ATT CTG TTC TGT GGC AGG TTC TCA TCT CCA TAA 1197  
 Lys Thr Lys Ser Ile Leu Phe Cys Gly Arg Phe Ser Ser Pro End  
 1198 AGCACATTTACTACATGGGGAGGTCCTCTCTCAGTATCTCCACCCTCAAAGCTCT 1257  
 1258 GTCAGGATGGCCAGTAGGGGGAAACCATGTTCCTCAAGATGAGGATCTTCTCTCCGCCA 1317  
 1318 GCCTGATCTTATGATGCCGTCATCAGCTCTCCCTATCTCGAATGCATTTTGCCCTTTT 1377  
 1378 CAGGTTATGTCTAATGATGCCATATAGCCCTTTGCTATGCCCGCTGATACTCATAACTTAT 1437  
 1438 AGCCAAATACTTACAGCCCTGGTAGAAGTCAATCAATGTGAAGAATGCTGGAGAGAAGGA 1497  
 1498 CAGATCTAGGAAAAGTTGCTGATCTTTCGAAAGGACACAGGGGACATTTCCCAACCCCA 1557  
 1558 ATGGCTCTTACAACTCTGGAATTTCCAGTTTCCACCCTGCTTCTGATGCTTTATTTG 1617  
 1618 GCTACTACGCGCCAGCATCTCTGATAGCATGTACATCACTCAAGTATTGACAGAAA 1677  
 1678 CARGCAATTTTAAATAAATAAATAAATTTAAGAGAGCTGCTCAAGTTCATTAATGG 1737  
 1738 TTGGATAATATCTGCTATTTGGCTCATCTCCATCAATTTCTTCTTCAAGTATG 1797  
 1798 ACAATTTATGGGTAAATCAGGCCACAGATCTTATTTTTCCTTTCACAAACACATGTA 1857  
 1858 ACCATTACTCAGTACTCTCCATCTCTCCCTCTGGTGTCTAACCACCAATCTCTCT 1917  
 1918 TTTATCTATGATAGTTTACCTATAGGAGCTTTTATACACATTTGCTTCTTGGCTTTCT 1977  
 1978 GATAATCTCTTTGATGCACACAGATGCTCTTTTCAGTGCAGTCTCTCTTATTTAATAC 2037  
 2038 TGTTCTGATCATGTGTATCCATGCCACAGCTAAGAATCTCCACCCTAAAATCAAGTACAG 2097  
 2098 AAATAACCTCTCTATGTTTTCCTCCAAAGCTTTCATGGATTGGGCTTATATATCTACG 2157  
 2158 TCATTTGTTCCAAATTTGAATTAATTAACATATACAGTATGGTGTAGGAGCCTGATGTGATT 2217  
 2218 CTTTATTTTGAACATCCAGTTTCCCAATACTGTAAATGTTTGAATCATGAAGTAA 2277  
 2278 TGCCCAACGAATAGGGGAAAGCAATGCTTCAACTACTGCTGGCTAGGCCAAGTACTCAAT 2337  
 2338 AGCCATTTGAACACTCTTACAAAGTGAAGAAAATATCAGGAATAACAGGAATGA 2397  
 2398 AGAAGGAGAGATTAATTTGTTCTGTAATCAGGGCCCTCAGAGAAGACTCAGTGGGGAG 2457  
 2458 ATGCTGCTGCCATGCCCTGACAACTGAGTTCATGTCTGGAACCTCCCATGGTGAAGAA 2517  
 2518 GAAACTTACTCTAGCAAGTTGTTCTGAGCTCCACCATGAACACCATACAAATAAAC 2577  
 2578 AAGCATTCCTGTAATCAGAAAATGATATACAGTAAGTAAATAATCAGAAAAAATACAC 2637  
 2638 ATAAAAAACCAATTTCTCTGCTGGAAGTTAATCTTTTAGGGAAGCCAGGAAGTAGAG 2697  
 2698 TAATAGAGCCCGTGAAGATGATAAAGTTAAGACAGGATGTACTCTCATCTCAGATG 2757  
 2758 TATCCGCATTCAGAAAGTCAATCCAGTATATCACTCACTGAAGTCCCAATAGGGC 2817  
 2818 AGTGACTTGAATATCTCTGAGCTATTTGACAGCAATTAAGAGGACCACTGACATG 2877  
 2878 ATCTCACACAGTAATAAGAAAATCATGTTTACACAGTAATAATGAATGGAGTACAGGA 2937  
 2938 GTTGTACTAGATAATTTATATTAATTTGATCAAGGTAGAGTCACTGGATAGGAAGGAG 2997  
 2998 CCTTACTTGAGAAATGCCCTCCATAACCTCTAGGGCAATTTTATAGTCATTTGATGTGAGAA 3057  
 3058 GCGTAGGCCAATTTGTCATAAGACAACCCCTATGTAGGTGGTCTCTGTTTAAAGGAAG 3117  
 3118 CAGGCTGAACAAGTCAGGGGAGCAAGCTAATAGTAAGCAGCACCCCTCTGTGGCCCGCT 3177  
 3178 CATCAGCTCTTCTCCAGCTCTTGTGACCTGTTTGTAGTCCCTTCTGACTCTCTCTCAAT 3237  
 3238 GATGAATAGTATAGTAAATGTAAGTCAATAAACATGATTTCCAAAAAATAAAAAA 3295

Fig. 1. Nucleotide and predicted protein sequence of raPIT5a. Arrows indicate the sequences which annealed to the degenerate oligonucleotide primers. Potential N-linked glycosylation sequences are boxed and a potential polyadenylation signal is underlined. A double line highlights the sequence used to prepare the glutathione S-transferase-raPIT5a fusion protein immunogen.

mained predominantly inside the cell, media from BSC-40 cells expressing raPIT5a were concentrated using a Nanospin microconcentrator (Gelman Sciences, USA). Cells were homogenised in 20 mM Tris-HCl, pH 7 at 4°C. 1/9 of the cell homogenate and 1/25 of the concentrated culture supernatant were fractionated on 10% SDS-polyacrylamide gels under reducing conditions using the buffer of Laemmli [15] and analysed by Western blotting. Polyclonal antibodies specific for raPIT5a were raised in rabbits using a raPIT5a-GST fusion protein immunogen prepared using sense and antisense PCR primers to sequences 841–860 and 940–957, respectively.

2.4. Complex formation between raPIT5a and granzyme B

raPIT5a was subcloned into pcDNA3.1 (Invitrogen) behind the T7 promoter. Radiolabelled raPIT5a was produced in a coupled TnT reticulocyte lysate system (Promega) using T7 RNA polymerase and incorporating [<sup>35</sup>S]methionine (Amersham). For complex formation 1 µl of the translation mix was incubated with varying amounts of human granzyme B in 67 mM Tris-HCl pH 7.6, 133 mM NaCl for 30 min at 37°C. Samples were separated on 10% SDS-polyacrylamide gels under reducing conditions using the buffer of Laemmli and analysed using a phosphorimager (Fuji FLA2000) or Western blotting using a monoclonal antibody specific for granzyme B [16].

3. Results

3.1. Nucleotide and predicted protein sequence of raPIT5a

The coding sequence of the raPIT5a cDNA is shown in Fig. 1. The predicted initiating methionine is located 73 bp from the 5' end of the DNA and is included in the single open reading frame of 374 amino acids ending with a termination

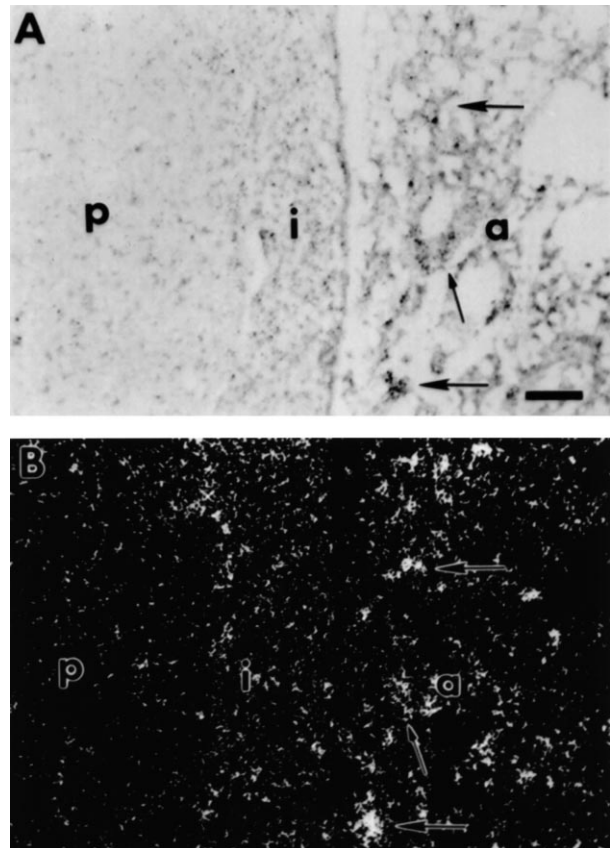


Fig. 2. Distribution of raPIT5a mRNA in the pituitary gland. Brightfield (A) and darkfield (B) images of the pituitary gland showing raPIT5a mRNA expression. The posterior (p), intermediate (i) and anterior (a) lobes are indicated. Arrows indicate selected cells in the anterior pituitary that are heavily labeled. The bar represents 200 µm.

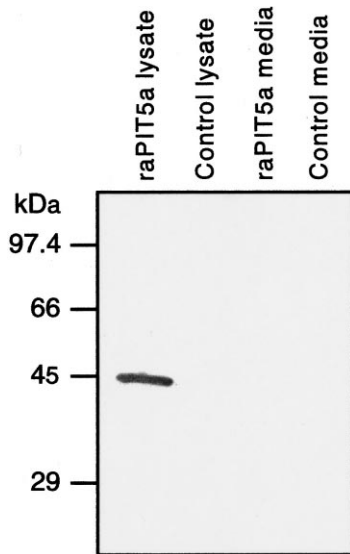


Fig. 3. Expression of recombinant raPIT5a in a monkey kidney cell line. Recombinant raPIT5a was transiently expressed in BSC-40 cells using the vaccinia virus/T7 RNA polymerase hybrid expression system. Cell lysate and culture medium from these cells and control cells that had been infected with vaccinia virus but not transfected with the raPIT5a cDNA expression plasmid were fractionated by SDS-PAGE and expression of raPIT5a detected by Western blotting using a raPIT5a-specific polyclonal antibody.

codon at nucleotide positions 1195–1197. raPIT5a lacks a typical cleavable NH<sub>2</sub>-terminal signal sequence but contains two hydrophobic regions near the amino terminus (amino acid residues 4–16 and 28–44) which are very similar to PAI-2 and PI-8. In PAI-2 these regions appear to enhance glycosylation and secretion [17]. A consensus polyadenylation signal is located at nucleotide positions 3267–3272, 10 bp before the poly(A) tail. The poly(A) tail supports the cloning of the complete raPIT5a cDNA and indicates a 3'-untranslated sequence of 1986 nucleotides. The protein has a predicted molecular mass of 42.3 kDa and potential N-linked glycosylation sites at Asn<sup>8</sup>, Asn<sup>23</sup>, Asn<sup>245</sup> and Asn<sup>359</sup>.

3.2. Cellular distribution of raPIT5a mRNA in the rat pituitary gland

raPIT5a mRNA was detected in the intermediate and anterior lobes of the pituitary (Fig. 2). Specific cells in the anterior pituitary were strongly labeled but their peptidergic phenotype has not been identified. Melanotropes in the intermediate lobe showed moderate labeling while no raPIT5a mRNA was detected in the posterior lobe. Identical distributions were seen with both probes. Sections probed with control sense ribo-probes showed no labeling (data not shown).

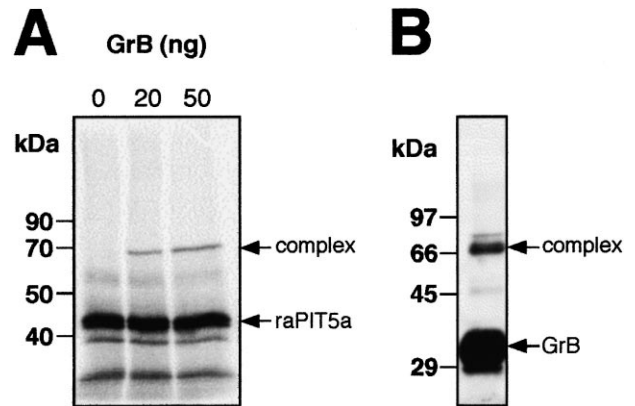


Fig. 4. Interaction between raPIT5a and human granzyme B. SDS-stable raPIT5a:granzyme B complexes were formed by incubating radiolabelled raPIT5a with varying amounts of granzyme B. Samples were separated by SDS-PAGE and detected using a phosphoimager (A) or by Western blot analysis with a granzyme B monoclonal antibody (B).

3.3. Expression of recombinant raPIT5a in BSC40 cells

To establish that the raPIT5a cDNA encodes a protein of the expected size and to determine if it was secreted the raPIT5a cDNA was expressed in the BSC-40 monkey kidney cell line. Recombinant raPIT5a protein was detected using a polyclonal antibody raised against amino acids 257–295 of raPIT5a expressed as a glutathione S-transferase fusion protein. A ~45-kDa anti-raPIT5a immunoreactive protein was detected in the cell homogenate of BSC-40 cells transfected with the raPIT5a cDNA (Fig. 3). No immunoreactive raPIT5a protein was detected in concentrated culture medium even after overexposure of the blot (data not shown). No immunoreactive protein was detected in cells or concentrated media that had been infected with vaccinia virus but not transfected with the raPIT5a cDNA.

3.4. Complexation of raPIT5a with human granzyme B

raPIT5a was expressed using a coupled in vitro transcription/translation system. Two major translation products were seen with molecular masses of ~43 and 44.5 kDa. The two raPIT5a proteins may result from alternative translation initiation at nucleotides 73 and 124, respectively. Incubation of raPIT5a with human granzyme B resulted in the formation of an SDS-stable complex. The composition of the complex was verified by the presence of labeled raPIT5a (phosphoimager, Fig. 4A) and granzyme B (Western blot with an anti-granzyme B monoclonal antibody, Fig. 4B).

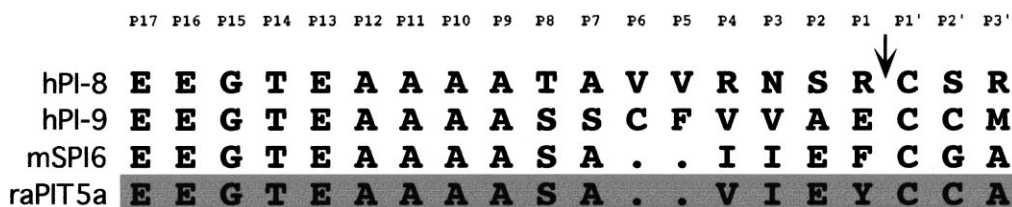


Fig. 5. Comparison of the reactive centre loop region of raPIT5a with other serpins. The reactive centre loop region of raPIT5a was compared with the reactive centre domains of human protease inhibitor 8 (hPI-8), human protease inhibitor 9 (hPI-9) and mouse SPI6. Sequences were aligned using the Genetics Computer Group sequence analysis software (v 6.0). The arrow indicates the putative P<sub>1</sub>-P<sub>1</sub>' bond.

#### 4. Discussion

We have identified a novel serpin, which is expressed in the pituitary. Comparison of the predicted coding sequence of raPIT5a with other protease inhibitors clearly identifies it as a member of the serpin superfamily. raPIT5a shows highest similarity to the recently cloned serpin cytoplasmic antiproteases PI-6 (58% identity, [18]), PI-8 (58% identity, [19]), PI-9 (70% identity, [19,20]) and SPI6 (86% identity, [20]). This places raPIT5a in the ovalbumin branch of the serpin superfamily. Overexpression of raPIT5a using a vaccinia-based expression system resulted in high levels of raPIT5a protein but no detectable secretion. Therefore, with respect to secretion, raPIT5a is probably more similar to PI-6 which has been suggested as a prototype for a new class of intracellular serpins [21].

Sequence conservation of the hinge region of the reactive centre loop of raPIT5a with other inhibitory serpins suggested raPIT5a has the potential for direct protease inhibition (Fig. 5). From PI7-P8 nine out of the ten amino acids are identical to the consensus sequence determined for inhibitory serpins [22]. The hypervariable reactive centre loop of serpins acts as a pseudosubstrate for the target protease and thus determines the specificity of the inhibitor. The P<sub>1</sub> residue is thought to be an important determinant of inhibitor specificity and is also used as a tool for classification of serpins. Sequence comparisons within the reactive centre loop region of different serpins are shown in Fig. 5. Alignment of the raPIT5a reactive centre loop with PI-8, PI-9 and SPI6 predicts P<sub>1</sub>-P<sub>1</sub>' residues as Tyr-Cys. In these alignments, a conserved cysteine in the reactive centre P<sub>1</sub>' position is identical to other putative cytoplasmic antiprotease enzymes and the viral antiprotease CrmA [18–20,23]. The most closely related serpins to raPIT5a that have been functionally characterised are human PI-9 and mouse SPI6, the latter hypothesised to be the functional homologue of human PI-9. Both these serpins are expressed in immune cells and inhibit the cytotoxic lymphocyte granule serine protease granzyme B. Inhibition requires a glutamic acid residue in the reactive centre loop [23]. Granzyme B induces apoptosis by activating members of the caspase enzyme family by cleavage after acidic amino acids [24]. As raPIT5a also has a glutamic acid in the reactive centre we investigated a possible interaction between raPIT5a and granzyme B and found raPIT5a formed an SDS-stable complex with human granzyme B.

Our results identify raPIT5a as a potential inhibitor of proteases which cleave target substrates following acidic amino acids. RaPIT5a could inhibit granzyme B or related proteases that may be expressed in the pituitary. Interleukin converting enzyme-like proteases, or caspases, which have been implicated in apoptosis [25] are also candidates for inhibition by raPIT5a. Caspases are synthesised as zymogens and activated by proteolytic cleavage at specific aspartic acid residues. Recently, similarities between the viral serpin CrmA and intra-

cellular serpins of the ovalbumin subfamily, particularly PI-9, have been identified [19] leading to the suggestion that PI-9 and homologues may inhibit apoptotic cysteine proteases [20,23].

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