Unique reactive site domains of neuroendocrine isoforms of alpha$_1$-antichymotrypsin from bovine adrenal medulla and pituitary revealed by molecular cloning

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Abstract Molecular cloning of bovine adrenal medulla (AM) and pituitary (Pit) α_1-antichymotrypsin cDNAs indicated novel isoforms of ACT. The deduced primary sequences indicated that the AM ACT and Pit ACT possess COOH-terminal reactive-site domains that are characteristic of serpins (serine protease inhibitors). Of high interest was the finding of unique reactive sites within AM ACT and Pit ACT which are predicted to possess Arg as P_1 residue. Arginine as P_1 residue parallels the cleavage specificity of neuroendocrine prohormone processing enzymes cleaving at basic residues. Furthermore, RT-PCR indicated tissue-specific expression of AM and Pit ACT mRNAs. The AM and Pit isoforms of ACT may regulate novel target proteases involved in neuroendocrine function.

Key words: α_1-Antichymotrypsin; Neuroendocrine; Serpin; P_1 residue; Prohormone processing enzyme

1. Introduction

Alpha$_1$-antichymotrypsin (ACT), a member of the serpin family of protease inhibitors, functions in plasma as a major acute phase protein whose levels are rapidly elevated in traumatic events including surgery [1], burn injuries [2,3], cancer [4], and disease [5]. More recently, evidence for neuroendocrine functions of ACT have been demonstrated. ACT is present in amyloid plaques of Alzheimer's Disease brains [6,7]. ACT is present within neurosecretory vesicles where it may potently inhibit a candidate prohormone processing enzyme known as PTP [8].

The structure of human liver ACT, deduced from the corresponding cDNA [9], indicates the presence of a reactive site domain in the COOH-terminal region of the protein, which is characteristic of members of the serpin family. Selectivity of ACT and other serpins for inhibiting target proteases is determined, in large part, by the reactive-site domain that contains the P_1–P_1' cleavage site [10,11]. It is known that the P_1 residue of the serpin resembles the cleavage specificity of the inhibited protease. Mutagenesis studies directed at altering the P_1 residue of ACT have demonstrated the importance of the P_1 residue in determining the specific protease that is inhibited [12]. Therefore, the P_1 residue may predict inhibited proteases based on parallel cleavage specificities.

The cloned liver ACTs in human [13] and bovine [9] have generally been thought to resemble ACT present in brain and endocrine tissues. However, recent characterization of a bovine pituitary ACT-like protein that inhibits a prohormone processing enzyme suggested that it was not identical to human liver ACT, since the pituitary ACT showed differential inhibitory potency and less sensitivity to detection by anti-ACT serum when compared to the liver ACT [9]. In addition, the existence of heterogeneous forms of ACT is supported by the presence of multiple bovine ACT genes in Southern analysis [9].

The goal of this study was to identify possible neuroendocrine isoforms of bovine ACT through sequence analysis of bovine AM and Pit ACT cDNAs that include the reactive-site domain. Molecular cloning of AM and Pit ACT cDNAs yielded clones possessing reactive-site domains with unique P_1 residues that differ from previously identified forms of ACT. These results demonstrate that neuroendocrine tissues express novel forms of ACT, suggesting regulation of target proteases in neuroendocrine function.

2. Experimental

2.1. RNA isolation

Total RNA was isolated from fresh bovine adrenal medulla, pituitary, and liver by the procedure of Chromoczynski [14]. One gram fresh tissue was minced, pulverized in liquid N_2, and solubilized in 10 ml of 4.0 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.1 M β-mercaptoethanol, and 0.5% *N*-sarkosyl. After addition of 2.0 M sodium acetate, pH 4.0 (1 ml) and phenol/chloroform extraction (by vortexing with 10 ml phenol and 2 ml chloroform/IAA 49/1 vol/vol, incubation at 4°C for 15 min, and centrifugation at 10,000 × *g* for 20 min), RNA in the aqueous phase was precipitated by isopropanol and resuspended in 10 ml Tris-HCl, 0.5 mM EDTA. Isolation of poly(A$^+$)RNA utilized oligo(dT) affinity chromatography, as described previously [9,15].

2.2. Molecular cloning of bovine AM and Pit ACT cDNAs

Bovine AM and Pit cDNA libraries were constructed in the lambda UniZAPXR vector (Stratagene), as described previously [15]. Molecular cloning of ACT cDNAs from bovine adrenal medulla and pituitary was accomplished by screening 1×10^6 recombinants from respective cDNA libraries with the bovine liver L-1 ACT cDNA (clone pHHK11), isolated by this laboratory [9]. The ^{32}P-labeled liver L-1 ACT cDNA (0.9 kb) (labeled by nick translation, according to the protocol by BRL) was hybridized to filter lifts of cDNA libraries in 6× SSC, 5× Denhardt's solution, 0.5% SDS, and 10 mg/ml salmon sperm DNA, at 60°C overnight. Filters were washed twice in 2× SSC, 0.1% SDS for 30 min at 60°C, and twice in 0.1× SSC, 0.5% SDS for 20 min at room temperature. After 3–4 rounds of screening, a single clone was obtained from

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Abbreviations: ACT, α_1-antichymotrypsin; AM, adrenal medulla; Pit, pituitary; serpin, serine protease inhibitor; PTP, prohormone thiol protease; RT-PCR, reverse transcription-polymerase chain reaction; 5′-RACE, 5′-rapid amplification of cDNA ends.

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each cDNA library. In vivo excision yielded the cDNAs in Bluescript KS-plasmid vector (according to the Stratagene protocol). DNA sequencing of both strands of the cDNAs was performed by the dideoxynucleotide chain-termination method [16] with the Applied Biosystems automated DNA sequencer, combined with manual DNA sequencing with the Sequenase version 2.0 sequencing kit (from USB). DNA and deduced amino acid sequences were analyzed by the MacVector and IntelliGenetics DNA-sequence software programs.

2.3. RT-PCR and Southern analysis

RT-PCR (reverse transcriptase-polymerase chain reaction) was utilized for detection of ACT mRNAs in bovine adrenal medulla, pituitary, and liver. RT-PCR with poly(A^+)RNA was performed with the GeneAmp thermostable rTth reverse transcriptase RNA PCR kit (Perkin Elmer), according to the manufacturer's protocol. PCR primers A (sense) and B (antisense) complementary to the AM ACT cDNA sequence were 5′-GGAAGAACGTCTTGAGAATCATTGTGC-

```
a
                                                    50                                                  100
CGGCACGAGC CAACTTCAGG GATTCTGAAG CTGCCAGGAG TCTAATAAAT GACTATGTGA AGAATAAAAC CCAGGGGAAA AAAGAAGAGC TGTTCAAGTA
  AlaArgAla AsnPheArg AspSerGlu AlaAlaArgSer LeuIleAsn AspTyrVal LysAsnLysThr GlnGlyLys LysGluGlu LeuPheLysTyr   33

                                                   150                                                  200
CCTTTCCCCG AGAACAGAGT TGGTGCTAGT GAATTACATC TACTTTAAAG CCCAATGGAA GACCCCCTTT GACCCCAAAC ACACTGAGCA GGCAGAGTTC
 LeuSerPro ArgThrGlu LeuValLeuVal AsnTyrIle TyrPheLys AlaGlnTrpLys ThrProPhe AspProLys HisThrGluGln AlaGluPhe    66

                                                   250                                                  300
CACGTGAGCG ACAACAAGAC GGTGGAGGTG CCCATGATGA CCCTTGACCT GGAAACCCCT TACTTCCGGG ACGAGGAGCT GGGCTGCACG CTGGTGGAGC
HisValSer AspAsnLysThr ValGluVal ProMetMet ThrLeuAspLeu GluThrPro TyrPheArg AspGluGluLeu GlyCysThr LeuValGlu     99

                                                   350                                                  400
TCACGTACAC CAGCAACGAC AGCGCCCTCT TCATCCTCCC CGACGAGGGC AAAATGCGGG ACCTGGAAGC CAAGCTGACC CCGGAGACGC TGACGAGGTG
LeuThrTyrThr SerAsnAsp SerAlaLeu PheIleLeuPro AspGluGly LysMetArg AspLeuGluAla LysLeuThr ProGluThr LeuThrArgTrp   133

                                                   450                                                  500
GCGAAACTCC CTGCAGCCCA GACGAATACA TGAACTCTAC CTGCCAAAAT TTTCCATCAA AAGCAACTAT GAACTGAATG ACATCCTCTC CCAGCTGGGC
 ArgAsnSer LeuGlnPro ArgArgIleHis GluLeuTyr LeuProLys PheSerIleLys SerAsnTyr GluLeuAsn AspIleLeuSer GlnLeuGly    166

                                                   550                                                  600
ATTAGGAAAA TATTTGCCAA CGCTGACTTG TCAGGAATCA CAGGGACCGC GGACCTGGTA GTCTCCCAGG TGGTCCACGG CGCTGCGCTG GACGTGGACG
 IleArgLys IlePheAlaAsn AlaAspLeu SerGlyIle ThrGlyThrAla AspLeuVal ValSerGln ValValHisGly AlaAlaLeu AspValAsp    199

                                              * 650            Primer A                                  700
                                             ---------------------------------->
 AGGAGGGCAC GGAAGGAGCT GCTGCCACGG GAATCAGCAT GGAAAGAACG ATCTTGAGAA TCATTGTGCG TGTCAACAGG CCCTTCCTGA TTGCCATAGT
GluGluGlyThr GluGlyAla AlaAlaThr GlyIleSerMet GluArgThr IleLeuArg IleIleValArg ValAsnArg ProPheLeu IleAlaIleVal   233

                                                   750                                                  800
 TCTCAAAGAC ACCCAGAGCA TCATCTTTTT GGGGAAAGTC ACCAACCCCA GTGAAGCCTAG AGCTCCGCTC TGAGCTGTGG GTCCCCTCCC TAAGGAAAAG
  LeuLysAsp ThrGlnSer IleIlePheLeu GlyLysVal ThrAsnPro SerGluAla***                                          252

                                                   850                                                  900
                                                                                                       <----
 GGTGCGCAGG TCCTGGGTGC AGCCTGGCCC CTGGGCACTG AGGATCCACC CTGCCTGTCC CCACCTGCTC ACCTCTGGGG AGGTGACAGG GACTCTGCTG

   Primer B                                        950                                                 1000
-------------------------
 GGAGCTCCCC ATGCACAGGG AGTCTGTGTC CGGGTCACAG GGAGACTCTG AAACCTCTCA CAGCAATAAA GCACTTTCCT CGGAAAAAAA AAAAAAAAAA

 AAA


b                    Primer C                       50                                                  100
    ---------------------------->
 GGCACGAGTC TCCCAGATGG GCATTAAGAA AATATTCACC GACGCTGACC TGTCAGGAAT CACAGGGACC GCGGACCTGG TAGTCTCCCA GGTGGTCCAC
  AlaArgVal SerGlnMet GlyIleLysLys IlePheThr AspAlaAsp LeuSerGlyIle ThrGlyThr AlaAspLeu ValValSerGln ValValHis    33

                                                   150                  *        Primer D                200
                                                                               <--------------------------------
 GGCGCTGCGC TGGACGTGGA TGAGGAGGGC ACGGAAGGTG CTGCTGCCAC GGGAATCGGC ATAGAAAGAA CGTTCTTGAG AATCATTGTG CGTGTCAACA
 GlyAlaAla LeuAspValAsp GluGluGly ThrGluGly AlaAlaAlaThr GlyIleGly IleGluArg ThrPheLeuArg IleIleVal ArgValAsn     66

                                                   250                                                  300
 GGCCCTTCCT GATTGCCGTA GTTCTCAAAG ACACCCAGAG CATCATCTTT TTGGGGAAAG TCACCAACCC CAGTGAAGCC TAGAGCTCCG CTCGAGCTGT
ArgProPheLeu IleAlaVal ValLeuLys AspThrGlnSer IleIlePhe LeuGlyLys ValThrAsnPro SerGluAla ***                 93

                                                   350                                                  400
 GGGTTCCCTC CCTAAGGAGC AGGGTTCCCA GATCCCTGGG TGCAGCCTGG CCCCTGGGCC CTGAGAATCC ACTCTGCCTG CCCCCACCTG CTCACCACTG

                                                   450                                                  500
 GAGAGGTGAC AGTGACTCTG CTGGGAGCTC TACATGCACA GGGAGTCTGT GTCCGGGTCA CAGGGAGACT CTGAAACCTC TCACAGCAAT AAAGCACTTT

 CCTCGGT
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Fig. 1. Nucleotide and deduced primary sequence of cDNAs isolated from bovine adrenal medulla (a) and bovine pituitary (b). The asterisk (*) indicates the predicted P_1 active-site residue. Potential Asn-Xaa-Thr(Ser) glycosylation sites are indicated by the underlined Asn. Consensus polyadenylation sequences are underlined. Nucleotides are numbered above the base sequences, and amino acids are numbered in the right hand margin. Arrows indicate the positions of primers A, B, C, and D used in RT-PCR in Fig. 4.

GTG-3′ and 5′-ACTCCCTGTGCATGGGGAGCTCCCAGCA-3′, respectively. PCR primers C (sense) and D (antisense) complementary to the Pit-ACT cDNA sequence were 5′-CGAGTCTCCCAGATGGGCATTAAGAA-3′ and 5′-CCTGTTGACACGCACAATGATTCTCAAGAACG-3′, respectively. First strand cDNA synthesis utilized 0.2 μg poly(A^+)RNA and antisense primer (B or D, at 0.15 μM) with the rTth DNA polymerase in the presence of $MnCl_2$ (according to the protocol by Perkin Elmer). PCR amplification of these cDNAs was then conducted by addition of $MgCl_2$ and sense primer (A or C, at 0.15 μM), and 35 PCR cycles, each cycle consisting of 1 min at 94°C, 1 min at 65°C, and 1 min 70°C, and a final step at 70°C for 7 min. PCR reaction products were analyzed by DNA agarose (2.5%) gel electrophoresis, as well as Southern blot analysis with ^{32}P-labelled (by nick-translation) AM ACT cDNA as probe. Filters for Southern blot hybridization were hybridized with ^{32}P-labelled AM ACT in 1% SDS, 10% dextran sulfate, 1.0 M NaCl overnight at 65°C. Filters were washed twice in 2× SSC at room temperature for 20 min, twice in 2× SSC containing 1% SDS at 65°C for 30 min, twice in 0.1× SSC at room temperature for 20 min, and subjected to autoradiography.

3. Results and discussion

3.1. Molecular cloning of ACT cDNAs from bovine adrenal medulla and pituitary

Screening of bovine AM and Pit cDNA libraries (1×10^6 recombinants) with the bovine liver L-1 ACT cDNA (clone pHHK11) [1], resulted in the isolation of partial ACT-like cDNAs that possess the reactive-site domain that is character-

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                                          #
b.AM ACT    ARANFRDSEA ARSLINDYVK NKTQGKKEEL FKYLSPRTEL 40
b.Pit ACT   ---------- ---------- ---------- ---------- 
b.L-1 ACT   ---------- ---------- ---------- ---------- 
b.L-2 ACT   LST..K.... .VK...E... ...H..I.K. .ND..VL.N.
h.L ACT     FATD.Q..A. .KK..... .G.R..ITD. I.DPDSQ.MM
                                  ^
                                                #
b.AM ACT    VLVNYIYFKA QWKTPFDPKH TEQAEFHVSD NKTVEVPMMT 80
b.Pit.ACT   ---------- ---------- ---------- ----------
b.L-1 ACT   ---------F RNS..... .......... ..........
b.L-2 ACT   I.L...F... ......N.N. .YES.....Q .ER.I.....
h.L ACT     ......F... K.EM....QD .H.SR.YL.K K.W.M....S
                       @      ^             @
                                     #
b.AM ACT    LDLET-PYFR DEELGCTLVE LTYTSNDSAL FILPDEGKMR 119
b.Pit ACT   ---------- ---------- ---------- ----------
b.L-1 ACT   .....-.... .......... .......... ..........
b.L-2 ACT   .Y...-.... .......... V.F.R..RG.. ....G...Q
h.L ACT     .HHL.I.... ....S..V.. .K..G.A... .....QD..E
                                        @
                                        &

b.AM ACT    DLEAKLTPET LTRWRNSLQP RRIHELYLPK FSIKSNYELN 159
b.Pit ACT   ---------- ---------- ---------- ----------
b.L-1 ACT   .......... .......... .......... ..........
b.L-2 ACT   .......... .......... .L..R.R.SR ...S.H.Q.K
h.L ACT     EV..M.L... .K...D..EF .E.G...... ...SRDYN..

b.AM ACT    DILSQLGIRK IFAN-ADLSG ITGTADLVVS QVVHGAALDV 198
b.Pit ACT   ARV..M..K. ..TD-..... .......... ..........
b.L-1 ACT   .......... ..G.-..... .......... ..........
b.L-2 ACT   ........K. ..TSD..F.. ..DDHKLAD. H.I.KPV...
h.L ACT     ...L....EE A.TSK..... ...ARN.A.. ....KVVS..

                              *
b.AM ACT    DEEGTEGAAA T-GISMERTI LRIIVR-V-- NRPFLIAIVL 234
b.Pit ACT   .......... .-..GI...F ......-.-- .......V..
b.L-1 ACT   .......... .-........ S.....-.-- ..........
b.L-2 ACT   G........V .AVVMATSS- .LHTLT-.SF .....LS.FC
h.L ACT     F.....AS.. .AVKITLLSA .VETRTI.RF .....MI..P

                            #
b.AM ACT    KDTQSIIFLG KVTNPSEA                         252
b.Pit ACT   .......... ........
b.L-1 ACT   .......... ........
b.L-2 ACT   .E......V. ........
h.L ACT     T...N.F.MS .....KQ.
                           +
                           @
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Fig. 2. Comparison of primary sequences deduced from bovine adrenal medulla, pituitary, and liver cDNAs. Comparison of ACT primary sequence homologies are illustrated for adrenal medulla (b. AM ACT), pituitary (b. Pit ACT), liver isoforms L-1 and L-2 (b. L-1 ACT and b. L-2 ACT, respectively) [9], as well as human liver ACT (h. L ACT) [19]. The position of the predicted P_1 residues is indicated by the asterisk (*). Dots represent identical residues; dashes indicate gaps or no sequence. Possible asparagine (N) glycosylation sites for b. AM ACT, b. Pit ACT, b. L-1 ACT, b. L-2 ACT, and h. L ACT are indicated by the symbols #, +, @, ^, and &, respectively.

istic of serpin protease inhibitors. One positive clone was isolated from each library, and clones were designated as pHHK13 for the AM ACT cDNA (AM ACT cDNA), and pHHK14 for the Pit ACT cDNA (Pit ACT cDNA). The observation that only one clone was isolated from each cDNA library indicated a relatively low abundance of these neuroendocrine ACT mRNAs. This prediction was confirmed by multiple attempts to obtain full-length clones through rescreening cDNA libraries with the liver ACT as probe, RT-PCR with different primers in 5′-RACE of isolated mRNA, and PCR of cDNA libraries. Detection of AM and Pit ACT mRNAs by PCR but not by Northern blots (section 3.3.) also suggests low abundance of mRNA. Importantly, however, these partial ACT cDNA clones indicate the expression of novel ACT isoforms in neuroendocrine tissues.

The determined nucleotide sequences and deduced primary sequences of these clones (Fig. 1) indicated that the AM ACT cDNA of 1.0 kb possessed an open reading frame of 252 amino acids, and the Pit ACT cDNA of 0.5 kb contained an open reading frame of 93 residues. Potential glycosylation sites of Asn-Xaa-Ser(Thr) [17] were present within the deduced primary sequence of AM ACT at residues #21, 71, 105, and 248, and were present within the deduced primary sequence of Pit ACT at residue #89.

Comparison of deduced primary sequences of the AM and Pit ACT cDNAs with the bovine liver L-1 and L-2 ACT cDNAs (encoded by clones pHHK11 and pHHK12, respectively, isolated by this laboratory [9]) indicated that these four ACT-like proteins possess varying degrees of homology (Fig. 2). The AM, Pit, and L-1 ACT isoforms share high degrees of homology with one another of 88–98%. However, these three ACT isoforms – AM, Pit, and L-1 – shared a lower degree of homology with the L-2 ACT of 67%, 53%, and 58%, respectively. Evidently, the identified neuroendocrine ACT cDNAs resemble the L-1 ACT more closely than the L-2 ACT. In addition, the bovine liver L-1 and L-2 forms, AM, and Pit isoforms of ACT share approximately 55–65% homology with the human liver ACT.

	P6	P5	P4	P3	P2	P1	P1′	P2′	P3′	P4′	P5′	P6′
b.AM ACT	G	I	S	M	E	R	T	I	L	R	I	I
b.Pit ACT	G	I	G	I	E	R	T	F	L	R	I	I
b.L-1 ACT	G	I	S	M	E	R	T	I	S	R	I	I
b. TI			S	R	E	R	R	T	I	R	I	I
b.L-2 ACT	V	V	M	A	T	S	S	-	L	L	H	T
b.EI	V	V	M	A	T	L	S	V	L	L	H	T
h. L ACT	V	K	I	T	L	L	S	A	L	V	E	T
m. cont.	I	G	G	I	R	K	A	I	L	P	A	V

Fig. 3. Alignment of the reactive-site domains among bovine ACT isoforms, human ACT, and mouse contrapsin. The reactive-site domains from P_6 to P_6' are compared for bovine adrenal medulla ACT (AM ACT), pituitary ACT (Pit ACT), liver ACT forms L-1 and L-2 (L-1 ACT, and L-2 ACT), bovine trypsin inhibitor (b. TI), bovine elastase inhibitor (b. EI), human liver ACT (h. L ACT), and mouse contrapsin (m. contrapsin). The alignments are arranged into groups possessing optimum homology. The identical residues within the same group are underlined. The arrow indicates predicted cleavage sites between P_1'–P_1'.

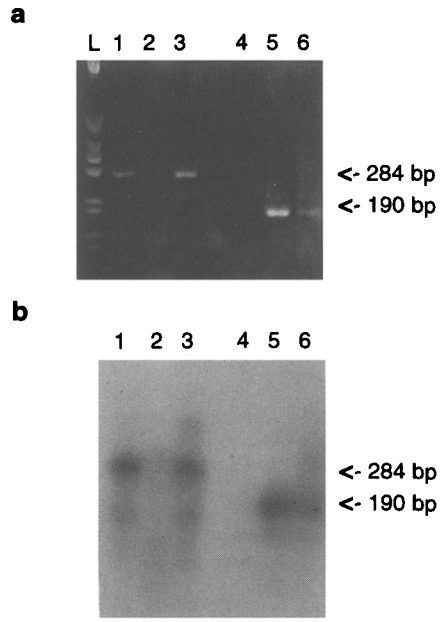

Fig. 4. RT-PCR and Southern analysis of ACT mRNAs. (a) Detection of AM and Pit ACT mRNAs by RT-PCR. RT-PCR with primers A/B complementary to the AM ACT cDNA is shown by ethidium bromide staining of DNA agarose gels (2.5%) (lanes 1–3) using poly(A^+)RNA templates from bovine adrenal medulla (lane 1), bovine liver (lane 2), and bovine pituitary (lane 3). RT-PCR with primers C/D complementary to the Pit ACT cDNA (lanes 4–6), utilized poly(A^+)RNA templates from bovine adrenal medulla (lane 4), bovine liver (lane 5), and bovine pituitary (lane 6). Standards from BRL's 1 kb ladder (lane L) shown are 1000, 510, 396, 344, 298, 220, 201, 154 and 134 bp. Relative positions of primers A–D with respect to AM and Pit ACT cDNAs are illustrated in Fig. 1a,b. (b) Southern analysis of RT-PCR of ACT isoforms. RT-PCR reactions in Fig. 4a were assessed in Southern blots with AM ACT cDNA as probe (as described in section 2).

3.2. *Analysis of reactive site domains of ACT isoforms*

The reactive site domains of the deduced primary sequences of the bovine neuroendocrine and liver ACT cDNAs were compared to the bovine plasma trypsin and elastase inhibitors [18], human liver ACT [13], and mouse contrapsin (the mouse counterpart of human or bovine liver ACT) [19]. Alignment of homologous sequences at the P_6 to P_6' positions of the reactive site domains indicated two subgroups of bovine ACT reactive sites that differ from human ACT and mouse contrapsin (Fig. 3). The first group is composed of the AM, Pit, and L-1 ACTs, and the bovine plasma trypsin inhibitor (TI). These serpins possess a typical consensus sequence for P_6 to P_6' residues similar to IGIERTILRII. The second group consists of the L-2 ACT and the bovine plasma elastase inhibitor (EI); these two serpins possess a similar reactive site sequence for P_6 to P_6' of VVMATXSXLLHT. The bovine ACT reactive site sequences clearly differ from those of human ACT and mouse contrapsin.

It is important to note the differences in the proposed P_1 residues within the bovine ACT reactive sites (Fig. 3). The P_1 residue of the serpin usually parallels the cleavage specificity of the target protease for its peptide substrate, with P_1–P_1' indicating the cleavage site. The isoforms of ACT from adrenal

Table 1
Homology of RT-PCR primers to ACT isoforms

Primer	Sequence (5'–>3')	ACT isoform	Percent homology among primer pairs
A	GGAAAGAACGATCTTGAGAATCATTGTGCGTG	AM/AM	100%
	A------------------------------	Pit/AM	97
	--------------C-----------------	L-1/AM	97
	--CT-CCT-ATCAC-CTTGCA--CGC-AAC--	L-2/AM	62
B	ACTCCCTGTGCATGGGGAGCTCCCAGCA	AM/AM	100%
	----------------------------	Pit/AM	100
	CTA-----------TA------------	L-1/AM	81
	T-TTTA-T---TGTTCT-TGGC---AGA	L-2/AM	25
C	CGAGTCTCCCAGATGGGCATTAAGAA	Pit/Pit	100%
	ATCC-------C----------G---	AM/Pit	77
	ATCC--------C-----------G-	L-1/Pit	77
	ATCC-T------C----T--C--G--	L-2/Pit	65
D	CCTGTTGACACGCACAATGATTCTCAAGAACG	Pit/Pit	100%
	------------------------------T--	AM/Pit	97
	-------------------------G------	L-1/Pit	97
	--------A--TG---G-T-GCG-GTGC--GA	L-2/Pit	56

Relative positions of primers A, B, C, and D to AM and Pit cDNAs are shown in Fig. 1a,b. Dashes indicate the same base sequences of ACT cDNAs compared with the primer sequence.

medulla and pituitary possess Arg as the proposed P_1 residue, suggesting that these ACTs may inhibit proteases cleaving at Arg residues. The predicted P_1 residue as Arg for these ACT isoforms is consistent with the known cleavage specificity of prohormone processing enzymes for basic residues [2–5]. Evidence supporting a possible role of neuroendocrine ACTs in controlling prohormone processing enzymes has been recently demonstrated in our studies showing inhibition of the 'prohormone thiol protease' (PTP) by the pituitary ACT-like protein [8]. Furthermore, purified adrenal medulla ACT-like protein inhibits PTP and the subtilisin-like PC1/3 and PC2 (PC = prohormone convertase) prohormone processing enzymes with differential potencies [20].

Most of the liver ACTs [9,13,18] from several species differ in their predicted P_1 residues compared to the AM and Pit ACTs (Fig. 3). The two bovine liver isoforms of ACT (L-1 and L-2) possess Arg or Ser as predicted P_1 residues, respectively [9]. The human ACT [14,16] and mouse contrapsin [19] contain Leu and Lys as P_1 residues, respectively. These differences in P_1 residues suggest that these isoforms of ACT may possess selectivity for inhibiting different target proteases. It will be important to analyze recombinant ACT isoforms in functional protease inhibitory assays.

3.3. RT-PCR analysis of ACT mRNAs

RT-PCR and Southern blotting were used to determine the presence of AM and Pit ACT mRNAs in bovine adrenal medulla, pituitary, and liver, since these mRNAs were not detected by Northern blots. Primers A and B complementary to AM ACT were designed to amplify a 284 bp DNA fragment that includes the reactive site domain. Analogously, primers C and D complementary to the Pit ACT should amplify a 190 bp DNA fragment containing the reactive site. RT-PCR with the primer pair A/B amplified a 284 bp band from adrenal medulla and pituitary, but not from liver (Fig. 4a); these bands were positive in Southern analysis with the AM ACT cDNA as probe (Fig. 4b). This result is consistent with primers A and B possessing 97% and 100% homology, respectively, with Pit ACT (Table 1). With respect to liver, primers A and B possess high homology with L-1 (97% and 81% homology, respectively), and low homology with L-2 (62% and 25% homology, respectively) (Table 1). Thus, if L-2 is the major form of ACT in bovine liver, RT-PCR with primers A/B may not generate a detectable DNA product, as indicated by results of these experiments (Fig. 4).

Primers C/D complementary to the Pit ACT cDNA possess high homology to both cloned AM and L-1 ACT cDNAs. Thus, RT-PCR would be predicted to generate a 190 bp DNA fragment from adrenal medulla, pituitary and liver. However, results indicate production of the 190 bp band from pituitary and liver, but not from adrenal medulla (Fig. 4). It is possible that small differences in nucleotide base sequence at the 5'- and 3'-end of the primers may not allow RT-PCR of AM ACT (Table 1). Another explanation for the lack of RT-PCR product from adrenal with primers C/D is based on our finding by Western blots of three distinct bands of ACT immunoreactivity in adrenal medulla [20], suggesting three isoforms of ACT. If the AM ACT cDNA obtained in this study is one of the minor isoforms of ACT in adrenal medulla, it may not be detected by RT-PCR, as shown by results of Fig. 4.

In conclusion, molecular cloning has revealed the presence of unique isoforms of ACT in the neuroendocrine tissues of bovine adrenal medulla and pituitary. These ACTs possess novel reactive-site domains compared to previously identified ACT serpin protease inhibitors. Of interest is the finding that the AM and Pit ACT isoforms possess Arg as the predicted P_1 residue. Prohormone processing enzymes, which are colocalized with ACT immunoreactivity in adrenal medulla and pituitary [8,20], possess specificity for cleavage at paired basic residues that include cleavage at Arg residues. The possibility that the neuroendocrine ACTs may inhibit prohormone processing enzymes should be examined in future studies. It will be important to determine the roles of ACT isoforms in neuroendocrine function.

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