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The Cystatin-Related Epididymal Spermatogenic Protein Inhibits the Serine Protease Prohormone Convertase 2

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The cystatin-related epididymal spermatogenic (CRES) protein is related to the family 2 cystatins of the cystatin superfamily of cysteine protease inhibitors. However, CRES lacks sequences important for cysteine protease inhibitory activity and is specifically expressed in reproductive and neuroendocrine tissues. Thus, CRES is distinct from cystatins and may perform unique tissue-specific functions. The purpose of the present study was to determine whether CRES functions as a protease inhibitor in *in vitro* assays. In contrast to mouse recombinant cystatin C, recombinant CRES did not inhibit the cysteine proteases papain and cathepsin B, suggesting that it probably does not function as a typical cystatin. CRES, however, inhibited the serine protease prohormone convertase 2 (PC2), a protease involved in prohormone processing in the neuroendocrine system, whereas cystatin C showed no inhibition. CRES did not inhibit subtilisin, trypsin, or the convertase family members, PC1 and furin, indicating that it selectively inhibits PC2. Kinetic analysis showed that CRES is a competitive inhibitor of PC2 with a K_i of 25 nM. The removal of N-terminal sequences from CRES decreased its affinity for PC2, suggesting that the N terminus may be important for CRES to function as an inhibitor. These studies suggest that CRES is a cross-class inhibitor that may regulate proprotein processing within the reproductive and neuroendocrine systems. (*Endocrinology* 144: 901–908, 2003)

THE CYSTATIN superfamily of cysteine protease inhibitors consists of three families, including the stefins, cystatins, and kininogens. Family 1 cystatins are 11,000 molecular mass intracellular proteins that lack disulfide bonds and include stefins 1 and 2. Family 2 cystatins, represented by cystatins C, D, S, and SA, are 13,000–14,000 molecular mass secretory proteins that have two characteristic intrachain disulfide bonds. The H- and L-kininogens, or family 3 cystatins, are large molecular mass secretory proteins that contain three family 2 cystatin domains, two of which possess inhibitory activity. All of the cystatin family members are potent ($K_i = 10^{-9}$–10^{-12} M) reversible inhibitors of the C1 family of cysteine proteases, including plant papain and the mammalian cathepsins B, H, L, and S (1). Mutagenesis and x-ray crystallography studies have identified three conserved regions in the cystatins that are important for their inhibition of papain-like cysteine proteases. These three regions include an N-terminal glycine, a glutamine-valine-glycine (Q-X-V-X-G) loop segment, and a second C-terminal hairpin loop containing proline-tryptophan (PW) residues, all of which participate in the formation of a wedge-shaped structure that blocks the active site cleft of cysteine proteases (1). As shown for cystatin C, the affinity and specificity of this interaction depend upon the presence of the N-terminal region (2, 3). Recently, cystatin C was shown to also inhibit mammalian legumain, a C13 family cysteine protease. The isolation of ternary complexes containing cystatin C, papain, and legumain suggested that binding and inhibition of legumain occur via a site distinct from the papain reactive site (4). Although the *in vivo* functions of cystatins are poorly understood, the biological significance of these proteins is made clear by various neurological diseases, such as hereditary amyloid angiopathy, which results from a single point mutation in the cystatin C gene (5), and familial epilepsy, which is caused by a mutation in the cystatin B gene (6).

The cystatin-related epididymal spermatogenic (CRES) protein defines a new subgroup within the family 2 cystatins by virtue of its low sequence identity (28%) with cystatin C but its conserved gene structure and cosegregation with the cystatin C gene to the distal region of mouse chromosome 2 (7). CRES also structurally resembles the cystatins by containing two putative intrachain disulfide bonds that dictate the tertiary structure of the proteins and by being a secreted protein. CRES, however, is not predicted to have the same biochemical activity as cystatins, because it possesses only the C-terminal PW site and lacks the two other consensus sites necessary for inhibition of C1 cysteine proteases (8). Furthermore, the N-terminal region of cystatin C responsible for its tight binding to cysteine proteases is poorly conserved in CRES, suggesting that the specificity of CRES differs from that of cystatin C. For these reasons, it has been difficult to predict whether CRES functions as a protease inhibitor or, alternatively, has acquired new noninhibitory functions as was shown for the cystatin-related protein fetuin (9).

CRES is also different from the cystatins in its reproductive- and neuroendocrine-specific expression. CRES is highly expressed in a region-dependent manner in the epididymis

Abbreviations: CRES, Cystatin-related epididymal spermatogenic; dNTP, deoxynucleotide triphosphate; His, histidine; MCA, methylcoumaryl amide; PC, prohormone convertase; SCCA, squamous cell carcinoma antigen; TBS-T, Tris-buffered saline containing 0.2% Tween 20.

(8), postmeiotic germ cells in the testis (10), and anterior pituitary gonadotroph cells (11). In addition, we recently detected expression in the ovary (12). Furthermore, we have shown that CRES is localized within the mouse sperm acrosomal cap (13) and the human sperm equatorial segment (14) suggesting that CRES may function during fertilization. The recent identification of testatin (15), cystatin T (16), and other CRES-like genes (12) and their high levels of expression in reproductive tissues indicate that the CRES subgroup of proteins is diverse and may have evolved to perform tissue-specific functions. Interestingly, these subgroup members are similar to CRES in that they are expected to structurally resemble cystatins, but because they possess only the C-terminal PW site, their functions are likely to differ. Like CRES, however, the functions of testatin and cystatin T are not known.

The prohormone convertases (PC) are calcium-dependent serine proteases with catalytic domains related to bacterial subtilisins. The seven mammalian members of the convertase family function within the secretory pathway, where they cleave proproteins at mono- or dibasic sites to generate mature proteins (17, 18). Several of the family members are ubiquitously expressed, whereas PC1, PC2, and PC4 are primarily found within the neuroendocrine and reproductive systems, respectively. Like other proteases, endogenous inhibitors probably control the activities of prohormone convertases. However, to date only two convertase inhibitors have been identified within the secretory pathway: 7B2, which inhibits PC2 at nanomolar concentrations (19), and pro-SAAS, which inhibits PC1 at high nanomolar concentrations (20–22). Therefore, it is likely that other convertase inhibitors exist.

The purpose of the present study was to determine whether CRES functions as a protease inhibitor in *in vitro* assays. In this report we demonstrate that CRES, although structurally belonging to the cystatins, is not an inhibitor of papain or cathepsin B, but, rather, is a novel cross-class inhibitor of the serine protease PC2. Our results suggest that CRES and perhaps other CRES-like proteins may mediate proprotein-processing events within the reproductive and neuroendocrine systems.

Materials and Methods

Animals and cell lines

Mature male and female CD-1 mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA), and were maintained under a constant 12-h light, 12-h dark cycle, with food and water *ad libitum*. All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guidelines for the Care and Use of Experimental Animals. The αT3-1 and LβT2 gonadotroph cell lines were gifts from Pam Mellon (University of California-San Diego, La Jolla, CA). GH_3 cells were obtained from American Type Culture Collection (Manassas, VA). The αT3-1 and LβT2 cells were cultured in 100-mm tissue culture dishes in DMEM with 4.5 mg/ml glucose, 5% fetal calf serum, 5% calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37 C in an atmosphere of 5% CO_2. GH_3 cells were maintained in DMEM with 10% fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Media and antibiotics were obtained from Invitrogen (Grand Island, NY), and serum was purchased from HyClone Laboratories, Inc. (Logan, UT).

Enzymes and substrates

Papain, trypsin (bovine pancreas), human cathepsin B, subtilisin, and aprotinin were purchased from Sigma-Aldrich (St. Louis, MO). PC1, PC2, and the proprotein convertase furin were prepared as described previously (23, 24). The substrates Boc-Phe-Ser-Arg-MCA and pGlu-Arg-Thr-Lys-Arg-MCA were purchased from Peptides International (Louisville, KY), whereas Z-Phe-Arg-MCA was obtained from Peninsula Laboratories, Inc. (San Carlos, CA), and Suc-Ala-Ala-Pro-Phe-MCA was obtained from Sigma-Aldrich.

Preparation of histidine (His) fusion proteins

A 366-bp CRES cDNA and a 306-bp cystatin C cDNA containing the coding sequence minus the signal peptide sequence were generated by PCR from plasmids containing the mouse CRES or cystatin C cDNAs, respectively. The amplified products were cloned into the pGEM-T-Easy vector (Promega Corp., Madison, WI), followed by restriction digestion and ligation into the pQE9 expression plasmid (QIAGEN, Chatsworth, CA), resulting in CRES or cystatin C sequences that are downstream of a 6-His tag. An N-terminally truncated CRES protein lacking the first 16 amino acids of the mature protein (CRESΔ1–16) was prepared as previously described (10). cDNAs were sequenced by the Texas Tech University Biotechnology Core facility to verify in-frame cloning with the His tag.

The His fusion proteins were expressed in *Escherichia coli* M15[pREP4] and purified under denaturing conditions from inclusion bodies by nickel affinity chromatography following the manufacturer's protocol (QIAGEN). To allow refolding of the proteins, the eluted fraction containing His-CRES or His-cystatin C fusion protein was dialyzed overnight at 4 C against 25 mM 2-(4-morpholino)-ethane sulfonic acid buffer (pH 5) containing 1 mM dithiothreitol, 5% glycerol, and 1 M guanidine, followed by changes into the same dialysis buffer containing 0.1 M guanidine, and then dialysis in buffer without guanidine. The protein amounts were quantified using the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL), concentrated by Centricon filters (cut-off, 3 kDa; Millipore Corp., Bedford, MA) if necessary, aliquoted, and stored at –20 C. A portion of the dialysis buffer from the final dialysis step was also aliquoted and stored at –20 C.

Enzyme assays

Trypsin assays were performed with 0.26 nM trypsin in 100 mM Tris buffer (pH 8), 10 mM $CaCl_2$, and 1 mM Boc-Phe-Ser-Arg-MCA. Papain (0.18 nM) and cathepsin B (39 nM) were assayed in 100 mM sodium phosphate buffer (pH 6), 1 mM dithiothreitol, and 2 mM EDTA with 20 μM Z-Phe-Arg-MCA. Subtilisin (0.25 nM) was assayed in 100 mM HEPES (pH 7.4), 0.5% Triton X-100, 1 mM $CaCl_2$, and 1 mM β-mercaptoethanol with 25 μM Suc-Ala-Ala-Pro-Phe-MCA. PC2 (0.7 nM) was assayed in 100 mM sodium acetate (pH 5), 0.1% Brij, and 1 mM $CaCl_2$ with 50 μM pGlu-Arg-Thr-Lys-Arg-MCA. Before use in the assays, pro-PC2 was diluted into 2× PC2 assay buffer and incubated at room temperature for 20 min to allow conversion to the active form (25). Reactions were initiated by the addition of substrate, and the enzymatically released MCA was measured using an Bowman fluorescence spectrophotometer (Aminco, Urbana, IL) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Continuous rates were measured for 100–240 sec, during which linear progress curves were observed for each enzyme tested. All reactions were carried out in an 80-μl reaction volume. To test various inhibitors, inhibitor and enzyme were diluted into the appropriate enzyme buffer, and reactions were initiated by the addition of substrate. To control for the possible effects of the recombinant protein buffer on enzyme activity, a volume of dialysis buffer equivalent to the volume of the His-CRES or His-cystatin C protein was added to enzymes assayed in the absence of inhibitor. In some experiments CRESΔ1–16 or CRES protein that had been heated at 100 C for 10 min was tested in inhibition assays of PC2.

To compare CRES inhibitory activities against the different prohormone convertase family members, increasing concentrations of His-CRES were combined with 7 nM PC2, 50 nM PC1, or 30 nM furin for 30 min at room temperature in a 96-well plate before the addition of 200 μM pGlu-Arg-Thr-Lys-Arg-MCA. PC1 was assayed in the same buffer as PC2. The assay for furin was performed using the same substrate as

for PC1 and PC2, but in 100 mM HEPES (pH 7), 5 mM $CaCl_2$, and 0.1% Brij (24). The K_m values of PC1, PC2, and furin were 11, 42, and 8 μM, respectively as determined using a computerized least squares fitting technique with EnzFitter (Biosoft, Cambridge, UK). Unless stated otherwise, the data for all assays are reported as a percentage of the control, with control representing enzyme activity in the absence of inhibitor (100%). All assays were performed three to five times, and the mean ± SEM for each point are presented.

Kinetics of PC2 inhibition by CRES

A kinetic analysis of CRES inhibition of PC2 activity was performed by incubation of 0.7 nM preactivated PC2 with increasing concentrations of His-CRES protein for 30 min at room temperature, followed by the addition of 25–100 μM pGlu-Arg-Thr-Lys-Arg-MCA. Enzyme activity was determined by a continuous rate measurement of substrate hydrolysis. Kinetic constants were determined using nonlinear least squares regression analysis of the raw data using the software package DynaFit (Biokin Ltd., Pullman, WA).

RT-PCR

Total RNA was isolated from mouse tissues and cell lines using TRIzol reagent (Invitrogen, Grand Island, NY) following the manufacturer's protocol. The RNA was quantitated by A_{260}/A_{280} and visualized by gel electrophoresis in 1% agarose gel containing borate buffer (pH 8.2) and 0.66 M formaldehyde. For RT-PCR, 2.5 μg RNA were incubated in RT reaction buffer containing 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3), 0.5 mM deoxynucleotide triphosphates (dNTPs), 20 U RNasin (ribonuclease inhibitor, Promega Corp.), and 2.5 μM oligo(deoxythymidine) (Promega Corp.) for 30 min at 37 C in the presence of 2.5 U ribonuclease-free deoxyribonuclease I (Roche, Indianapolis, IN). After heat inactivation of deoxyribonuclease I at 75 C for 5 min, an aliquot was reserved for PCR amplification as a no RT control to ensure the removal of all DNA. Moloney murine leukemia virus reverse transcriptase (50 U; Roche) was added to the remainder, and the reactions were incubated at 42 C for 60 min, 99 C for 5 min, and 5 C for 5 min.

Two and a half microliters of each RT and no RT reaction were amplified by PCR in separate reactions using primers recognizing PC2, CRES, and S16 cDNAs. S16 RNA was amplified as a constitutive control to measure the relative efficiency of each RT reaction. PCR master mixes containing 10 mM Tris (pH 8.3), 50 mM KCl, 0.5 μM each of forward and reverse primers, and 1.25 U *Taq* DNA polymerase (Fisher Scientific, Pittsburgh, PA) were prepared for each set of PCR primers so that all compared PCRs were from the same master mix. $MgCl_2$ and dNTP concentrations were optimized for each set of primers. PC2 PCRs were carried out in 1.5 mM $MgCl_2$ and 0.14 mM dNTPs for 40 cycles. CRES reactions consisted of 2.2 mM $MgCl_2$ and 0.19 mM dNTPs for 40 cycles, and S16 reactions were amplified using 2 mM $MgCl_2$ and 0.14 mM dNTPs for 25 cycles. The cycling parameters consisted of 45 sec at 95 C for denaturing, 25 sec at annealing temperature for each primer set, and 1 min at 72 C for extension, after which the reactions were incubated at 72 C for 7 min using a minicycler (MJ Research, Inc., Watertown, MA). RT-PCR products were analyzed by electrophoresis on 1.5% agarose/1× Tris/acetate/EDTA gels. RT-PCR products generated with each primer pair were cloned into the pGEM-T-Easy vector (Promega Corp.) and sequenced to confirm identities.

Oligonucleotide primers

PCR primers (Invitrogen, La Jolla, CA) were designed from the known sequences for mouse PC2, CRES, and S16 cDNAs using PrimerSelect 5.0 (DNASTAR, Madison, WI): PC2 sense, 5′-GGC GGC CGG GTT CCT CTT CT-3′; antisense, 5′-GTT CCA TCG GCT TGC CCA GTG TT-3′ (annealing temperature, 59 C); CRES sense, 5′-CAA GGA AAG TGA GGA CAA ATA TGT C-3′; antisense, 5′-GTG ACA GAC TTG AAC CAC AGG TT-3′ (annealing temperature, 64 C); and S16 sense, 5′-CGC TGC AGT CCG TGC AGG TGT T-3′; antisense, 5′-TCC AAA CTT TTT GGA TTC GCA GCG-3′ (annealing temperature, 57 C).

Western blot analysis

The αT3-1 cells were grown to confluence and scraped into buffer containing 25 mM Tris (pH 7.4), 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 0.5 mM *p*-chloromercuriphenylsulfonic acid, and 10 mM iodoacetamide. Cells were lysed by Polytron (Brinkmann Instruments, Inc., Westbury, NY), and lysates were centrifuged at 12,000 × *g* to remove insoluble material. Mouse testis and pituitary tissue were lysed by Polytron in buffer containing 20 mM Tris (pH 7.4), 50 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholic acid, 0.5% sodium dodecyl sulfate, 0.5% aprotinin, 1 mM EDTA, and 8.6 mM iodoacetamide, followed by centrifugation at 12,000 × *g*. Protein concentrations were determined by the bicinchoninic acid reagent (Pierce Chemical Co.).

Ten to 40 μg protein were separated on 10% (PC2) or 15% (CRES) SDS-PAGE gels under reducing conditions, followed by transfer to polyvinylidene difluoride membrane (Millipore Corp.). Blots were incubated for 1 h with Tris-buffered saline [50 mM Tris-HCl (pH 7.4) and 200 mM NaCl] containing 0.2% Tween 20 (TBS-T) and 3% (wt/vol) nonfat dry milk at room temperature, followed by incubation with a polyclonal rabbit antimouse PC2 peptide antiserum (1:4,000) (26) or an affinity-purified polyclonal rabbit antimouse CRES antibody (0.2 μg antibody/cm^2 membrane) at 4 C overnight. The blots were washed three times for 10 min each time in TBS-T and incubated with a goat antirabbit secondary antibody conjugated to horseradish peroxidase (Biosource Technologies, Inc., Camarillo, CA) at 1:20,000 for 2 h at room temperature. The blots were washed extensively in TBS-T, followed by 20 min in TBS, incubated with Supersignal reagent (Pierce Chemical Co.) for 5 min, and exposed to film.

Results

Analysis of CRES inhibitory activity

Studies were first carried out to determine whether CRES functions as a cystatin and inhibits the cysteine proteases papain and cathepsin B. Recombinant mouse His-CRES protein was incubated with papain or cathepsin B and the appropriate substrate, followed by an immediate and continuous measurement of the rate of substrate hydrolysis by the enzyme. Recombinant mouse His-cystatin C prepared in an identical manner as CRES was tested in separate reactions as a positive control. Cystatin C inhibited both papain and cathepsin B, whereas CRES in concentrations up to 6 μM showed no inhibitory activity against these cysteine proteases (Fig. 1). Because CRES is expressed at sites of proprotein processing, and the CRES sequence contains several monobasic and dibasic sites, we next tested whether CRES could inhibit proprotein-processing enzymes, specifically prohormone convertases. Incubation of CRES with PC2 resulted in a dramatic decrease in the rate of substrate hydrolysis by PC2 compared with activity in the absence of CRES (Fig. 1). In contrast, cystatin C did not inhibit PC2 activity, demonstrating the specificity of the CRES/PC2 interaction. CRES did not inhibit the related serine protease subtilisin or trypsin, a serine protease with broad substrate specificities, supporting the idea that CRES is a specific inhibitor of PC2 and not a general inhibitor of serine proteases (Fig. 1). CRES also did not inhibit the convertase family members PC1 and furin, further indicating that CRES may be a specific inhibitor of PC2 (Fig. 2).

CRES is a competitive inhibitor of PC2

A double-reciprocal (Lineweaver-Burk) plot of initial velocities against substrate concentration is shown in Fig. 3. From this and the results of nonlinear least squares regression analysis of different inhibition types using the software package Dynafit (Biokin Ltd.), we concluded that CRES is a competitive inhibitor of PC2 with a K_i of 25 ± 3.1 nM.

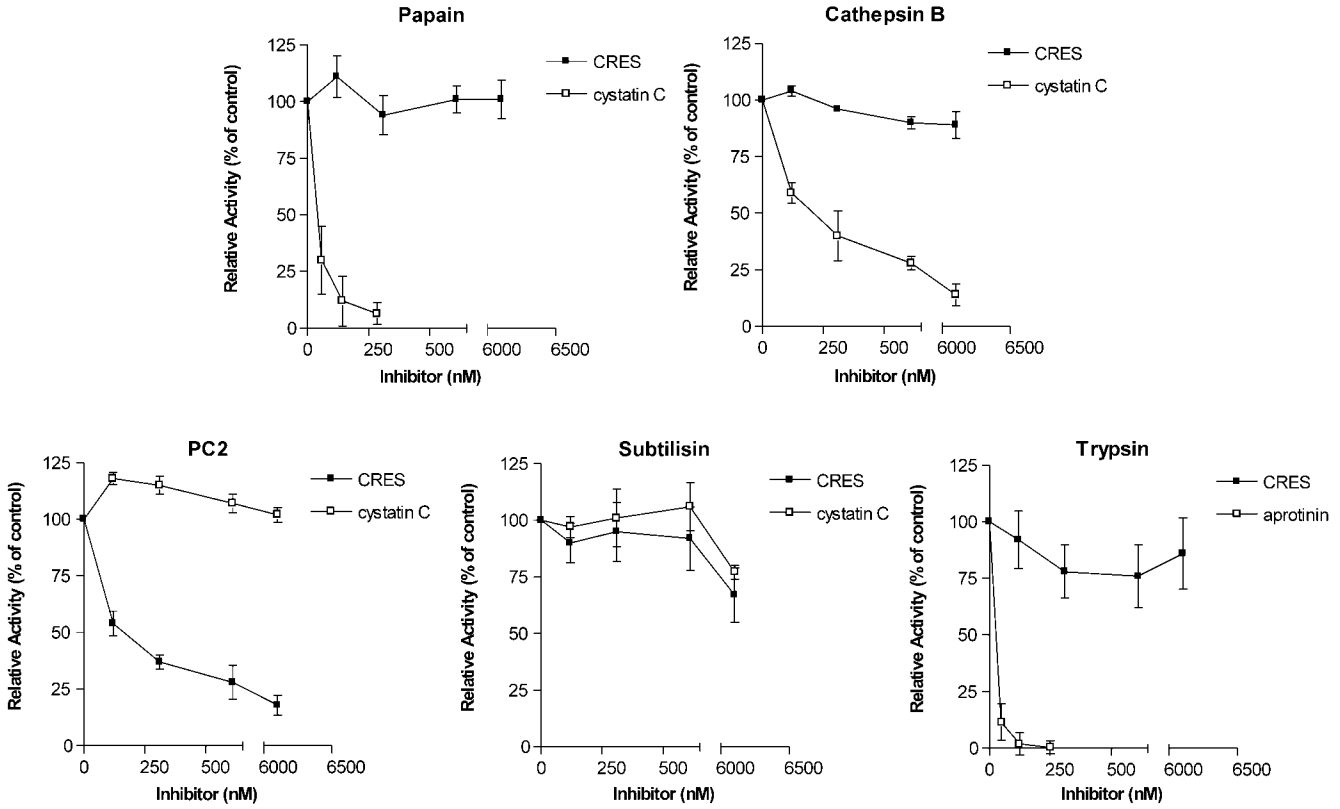

FIG. 1. Analysis of CRES inhibitory activity. Increasing concentrations of His-CRES (120–6100 nM) were combined with 0.18 nM papain and 20 μM Z-Phe-Arg-MCA, 39 nM cathepsin B and 20 μM Z-Phe-Arg-MCA, 0.26 nM trypsin and 1 mM Boc-Phe-Ser-Arg-MCA, 0.25 nM subtilisin and 25 μM Suc-Ala-Ala-Pro-Phe-MCA, or 0.7 nM PC2 and 50 μM pGlu-Arg-Thr-Lys-Arg-MCA in the appropriate enzyme buffers, followed by immediate measurement of enzyme activity in a continuous rate assay. Positive controls for the enzyme assays included the addition of equimolar concentrations of His-cystatin C or aprotinin, known inhibitors of cysteine proteases or trypsin, respectively. Data represent the mean ± SEM of three replicates.

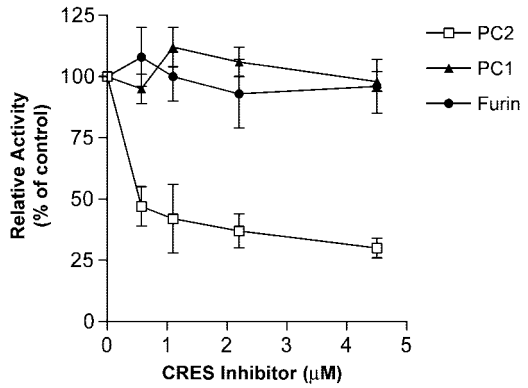

FIG. 2. Specificity of CRES inhibition of prohormone convertase family members. Increasing concentrations of His-CRES were combined with 7 nM PC2, 50 nM PC1, 30 nM furin, and 200 μM pGlu-Arg-Thr-Lys-Arg-MCA in the appropriate enzyme buffer, followed by measurement of enzyme activity. Data represent the mean ± SEM of three replicates.

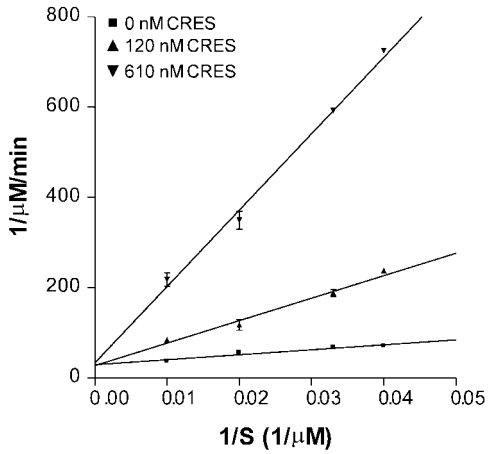

FIG. 3. Double reciprocal plot of CRES inhibition of PC2. Increasing concentrations of His-CRES (120–610 nM) were combined with 0.7 nM PC2 for 30 min at room temperature before the addition of 25–100 μM pGlu-Arg-Thr-Lys-Arg-MCA in assay buffer and the immediate measurement of enzyme activity in a continuous rate assay. The data were analyzed by the DynaFit program, and the initial velocities *vs.* substrate concentration are shown. ■, PC2 activity in the absence of CRES inhibitor (control); ▲, 120 nM CRES; ▼, 610 nM CRES.

Characterization of CRES protein inhibition

Several studies have demonstrated the importance of the N-terminal region in cystatin C for high affinity binding to cysteine proteases (2, 3). To determine whether the N terminus of CRES is important for its inhibitory activity, we measured PC2 activity in the presence of CRESΔ1–16. As shown in Fig. 4A, the truncated protein was less effective as a PC2 inhibitor than the full-length protein, implying that the N-terminal sequences are important for CRES inhibitory ac-

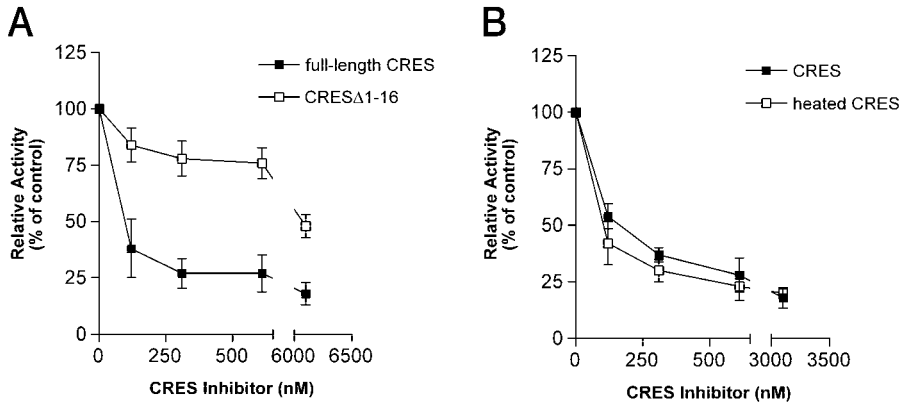

FIG. 4. Effect of N-terminal truncation or heat on CRES inhibition of PC2. A, Increasing concentrations of full-length or CRESΔ1–16 were combined with 0.7 nM PC2 and 50 μM pGlu-Arg-Thr-Lys-Arg-MCA, followed by measurement of PC2 activity in a continuous rate assay. B, Varying concentrations of His-CRES or His-CRES previously heated to 100 C for 10 min were combined with 0.7 nM PC2 and 50 μM pGlu-Arg-Thr-Lys-Arg-MCA, followed by measurement of PC2 activity in a continuous rate assay. Data are the mean ± SEM of three replicates.

tivity. At this time, however, we cannot rule out the possibility that the deletion of 16 amino acids could also induce a major structural change in the CRES protein that subsequently could affect its inhibitory activity. The heat stability of CRES was also assessed by heating CRES protein for 10 min at 95 C and then examining its inhibitory activity against PC2. Heat-treated CRES inhibited PC2 no differently than untreated CRES, suggesting that CRES is a stable protein and resistant to inactivation by heat (Fig. 4B).

Colocalization of CRES and PC2

To determine whether PC2 and CRES are expressed in the same tissues, RT-PCR was performed on various mouse tissues and anterior pituitary gland cell lines. As shown in Fig. 5A, both *Cres* and PC2 mRNAs were detected at varying levels in mouse testis, epididymis, ovary, and pituitary as well as in the αT3-1 gonadotroph cells. PC2 was also detected in the somatotroph/lactotroph cell line GH_3, but surprisingly was not detected in LβT2 cells, a gonadotroph cell line that expresses both the α- and β-subunits of LHβ (27). Because LβT2 cells represent a more differentiated gonadotroph cell than αT3–1 cells, which express only the α-subunit (28), the lack of PC2 expression could reflect the absence of specific hormonal stimuli. Studies are currently in progress to determine whether the administration of steroid hormones and/or GnRH will induce PC2 mRNA expression in LβT2 cells. Alternatively, the lack of PC2 expression in these cells could be a result of the simian virus 40-mediated cell transformation used to generate the cell line (27).

Because *Cres* and PC2 mRNAs were coexpressed in the αT3-1 cells, Western blot studies were next carried out to examine protein. Analysis of αT3-1 cell extracts showed the presence of the 68-kDa PC2 protein as well as small amounts of the proform of PC2 (Fig. 5B). Both PC2 proteins were blocked when the antibody was preincubated with immunizing peptide, indicating the specificity of the antibody (data not shown) (26). We have previously shown that there are several forms of CRES protein in the epididymis and testis, including a predominant 19-kDa *N*-linked glycosylated protein and a 14-kDa nonglycosylated protein as well as a 29-kDa protein that may represent a sodium dodecyl sulfate-stable CRES protein dimer (Fig. 5B) (10, 11, 13). As shown in Fig. 5B, CRES protein was also expressed in αT3-1 cells. The majority of CRES was present as the 29-kDa protein, previously identified as the major CRES form in the

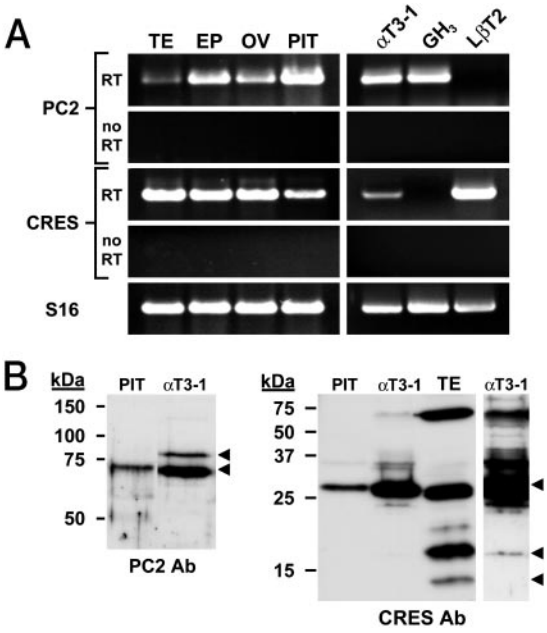

FIG. 5. Coexpression of CRES and PC2 in mouse tissues and cell lines. A, RT-PCR analysis of *Cres* and PC2 mRNA in mouse TE, testis; EP, epididymis; OV, ovary; PIT, pituitary gland; αT3-1 and LβT2 gonadotroph and GH_3 somatotroph/lactotroph cell lines. no RT, PCRs carried out in the absence of RT to ensure the PCR products observed in the presence of RT were not due to contaminating DNA. An aliquot of the same PCR master mix was used for amplification of the constitutive ribosomal protein gene S16 to confirm the presence of RNA in each tissue sample. B, Western blot analysis of PC2 (*left panel*) and CRES (*right panels*) proteins. Seven and 20 μg of protein from whole mouse pituitary gland (PIT) and αT3-1 gonadotroph cell extracts, respectively, were examined for PC2 protein, whereas 50, 40, and 10 μg protein from mouse pituitary gland (PIT), αT3-1 cells, and mouse TE, respectively, were examined for CRES protein on reducing SDS-PAGE gels. *Arrows* indicate the presence of the pro- and mature forms of PC2, and the 14-, 19-, and 29-kDa forms of CRES. A longer exposure of the αT3-1 lane of the CRES Western blot is also shown to demonstrate the presence of the 19-kDa CRES protein.

LβT2 cells and pituitary gland (11). Longer exposure of the Western blot also revealed low levels of the 19-kDa monomeric CRES protein in the αT3-1 cell extracts, whereas the 14-kDa CRES protein was not detected. The higher molecular mass protein of approximately 60 kDa present in the testis and at much lower levels in αT3-1 cells may represent a cross-reacting epitope or a sodium dodecyl sulfate-stable

CRES protein complex. All of the aforementioned CRES proteins disappeared or were dramatically reduced after incubation of similar blots with an antiserum depleted of CRES antibody, thus indicating that they represent specific interactions (data not shown).

Discussion

A hallmark of all cystatins is their potent inhibition of C1 cysteine proteases such as papain and cathepsins. Our data demonstrate that CRES does not appear to function as a typical cystatin, as it did not inhibit papain or cathepsin B. These observations were not surprising given that CRES lacks N-terminal sequences and a key Q-X-V-X-G sequence that together participate in blocking the active site of C1 cysteine proteases. In contrast to its lack of inhibition of cysteine proteases, the studies presented herein demonstrate that CRES is an inhibitor at nanomolar concentrations of the serine protease PC2. Although other convertase family members, such as PC4, PC5/6, PACE4, and PC7/8, need to be tested, the fact that CRES did not inhibit PC1, furin, or the related serine protease subtilisin suggests that CRES inhibitory activities against PC2 are specific and not the result of a general inhibition of serine proteases.

An analysis of the kinetics of the CRES/PC2 interaction revealed competitive inhibition, indicating that CRES binds to the enzyme active site. Thus, CRES has a mechanism of action similar to that of 7B2 and pro-SAAS, which are competitive inhibitors of PC2 and PC1, respectively (20, 21, 29). PC2 inhibition by the 7B2 CT peptide has been previously shown to require a highly conserved VNPYLQG site in addition to a dibasic pair (30). The fact that a form of CRES lacking 16 amino acids from the N terminus inhibits PC2, albeit at greatly reduced levels, implies that the inhibitory region is located elsewhere. The C-terminal portion of CRES contains several pairs of basic residues (Fig. 6). Of these, the last pair (Lys^{115}-Lys^{116}, mouse, rat; Arg^{115}-Lys^{116}, human) is well conserved; in addition, the P4 aliphatic hydrophobic residue (Leu^{113}), previously found to be important for inhibition of PC2 by the 7B2 CT peptide (31, 32), is present. Mutagenesis experiments will be required to define the actual sequences within CRES, including those within the N terminus, that participate in the inhibition of PC2; such studies are now in progress.

A comparison of CRES with the convertase inhibitors identified to date reveals little sequence similarity between the proteins, and therefore it appears that convertase inhibitors are not from a single gene family. However, 7B2 and pro-SAAS are structurally similar in containing a furin consensus sequence (R-X-X-R) approximately 30–40 residues from the C terminus, a proline-rich region in the middle of the protein, and a C-terminal inhibitory peptide (21). In the first two respects, CRES is not similar to 7B2 and pro-SAAS. However, CRES does contain di- or multibasic sites within the C-terminal region, and because there is some inhibitory activity in the absence of the N-terminal region, it is likely that the C terminus contains an inhibitory peptide.

Taken together, our studies suggest that CRES functions as a novel cross-class inhibitor with a structural relationship to the cystatins, but a functional relationship to serine protease inhibitors or serpins. Although there are several examples of cross-class inhibition between families of cysteine and serine proteases, most are of inhibition of cysteine proteases by serpins. For example, squamous cell carcinoma antigen 2 (SCCA2) is 92% identical to SCCA1, a well-characterized serpin; however, it does not inhibit chymotrypsin, a traditional serpin target, but, rather, inhibits the cysteine proteases cathepsins L and S (33). More recently, SQN-5, a serpin similar to SCCA1 and -2, was shown to exhibit dual mechanistic class inhibition by inhibiting both serine and cysteine proteases (34). Another example of cross-class inhibition has been identified within the apoptotic pathway. The viral cowpox protein crmA, a serpin, is a potent inhibitor of the IL-1B-converting enzyme, a cysteine protease (35). In addition, the serpin protease nexin2 has been shown to inhibit prohormone thiol protease, a cysteine protease (36). Finally, a synthetic peptide representing a domain of cystatin SA that contains the PW motif conserved in CRES and CRES-related proteins was shown to possess inhibitory activity against both cysteine and serine proteases (37), suggesting that, like serpins, some cystatins may also interact with other families of proteases. Indeed, our observation that CRES inhibits a serine protease rather than a cysteine protease, is one of the first examples of a cystatin that has acquired new protease inhibitory functions and exhibits cross-class inhibition of a serine protease. Most serpins inhibit their target proteases by forming a 1:1 stochiometric complex with the active site of the protease, resulting in sodium dodecyl sulfate-stable complexes (38). CRES does not appear to inhibit by the same mechanism as serpins, because preliminary analyses using recombinant proteins failed to reveal higher molecular mass complexes of CRES and PC2 by SDS-PAGE (Cornwall, G. A., *et al.*, unpublished observations). Also, the kinetics of the CRES/PC2 interaction do not support the mechanism of irreversible protease inactivation exhibited by serpins. Fi-

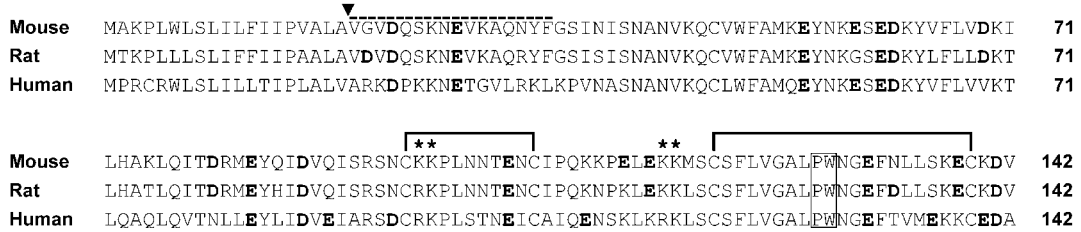

FIG. 6. Mouse, rat, and human CRES protein sequences. Acidic residues Asp (D) and Glu (E) are in *bold*, conserved di- and multibasic sites are indicated by an *asterisk*. The two hairpin loop structures formed by disulfide bond formation and conserved in cystatin family 2 members are indicated by the *brackets*. The PW site is marked by a *box*. The *arrowhead* indicates the predicted signal peptide cleavage site, and the 16 amino acids missing from mouse CRESΔ1–16 are shown by the *dashed line*.

nally, we cannot rule out that CRES may inhibit members of other families of cysteine proteases. However, preliminary studies indicate that CRES does not inhibit caspase-3, a member of the C14 family of cysteine proteases (Hsia, N., and G. A. Cornwall, unpublished observations), and it is also unlikely that CRES inhibits the C13 cysteine protease, legumain, because it lacks the Asn^{39} residue that is thought to be important for legumain binding by cystatin C (4). Mammalian homologs for most of the other families of cysteine proteases have yet to be identified.

Our data showing that CRES functions as a protease inhibitor, and in particular as a cross-class inhibitor of the serine protease PC2, represent a critical first step toward understanding the biological roles that CRES may perform in the neuroendocrine and reproductive systems. The K_i values we observed are low enough to suggest that CRES inhibition of PC2 is physiologically relevant. Indeed, our previous studies showing the colocalization of CRES with LHβ in anterior pituitary gonadotroph cells *in vivo* and the presence of CRES in granular extracts from LβT2 cells (11), taken together with other reports showing that PC2 is expressed in gonadotroph cells (39, 40), suggest that within the pituitary gland CRES and PC2 may interact. In support of this, our RT-PCR and Western blot studies show coexpression of CRES and PC2 mRNA and protein in αT3-1 gonadotroph cells. The role of CRES as a PC2 inhibitor in the testis and epididymis is less clear. PC2 has been shown previously to be expressed in the epididymis (41), and our RT-PCR studies confirm this. However, the epididymal cell population expressing PC2 has not been determined. In the testis the PC4 family member appears to be the predominant convertase and is present in round spermatids (42), where CRES is also localized, whereas in the ovary PC1/3 and PC4 are expressed in the corpus luteum (43, 44), where we have recently also detected *Cres* mRNA (12). Our RT-PCR analyses show, however, that PC2 mRNA is also present in the testis and ovary, and further studies are needed to identify the relevant cell populations. At this time it remains possible that CRES may perform distinct functions in different tissues and consequently may inhibit other convertase family members in these tissues. Alternatively, CRES may inhibit PC2-like proteases that have yet to be identified. Studies are currently in progress to examine CRES inhibition of other convertase family members as well as to assess PC2 expression in the epididymis, testis, and ovary. In addition, biochemical approaches are being used to examine CRES protein interactions *in vivo*. Taken together, the studies presented herein are significant in that they are the first to identify a function for a member of a growing family of cystatin-related proteins.

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