Structure and Transcriptional Regulation of the Human Cystatin A Gene

THE 12-O-TETRADECANOYLPHORBOL-13-ACETATE (TPA) RESPONSIVE ELEMENT-2 SITE (−272 TO −278) ON CYSTATIN A GENE IS CRITICAL FOR TPA-DEPENDENT REGULATION

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Cystatin A, a cysteine proteinase inhibitor, is one of the precursor proteins of cornified cell envelope of keratinocytes and is expressed during the late stage of keratinocyte differentiation. We have isolated and characterized the human cystatin A gene. The cystatin A gene consists of three exons and two introns. The first, the second, and the third exons consist of coding sequences that are 66, 102, and 126 base pairs in length, respectively. The first and the second introns consist of 14 and 3.6 kilobase pairs, respectively. The transcription initiation site was located 55 base pairs upstream from the first translation site. The fragment, +77 to −2586 in the 5′-flanking region of the human cystatin A gene, was subcloned into a chloramphenicol acetyltransferase (CAT) reporter vector. The expression vector, p2672CAT, produced a significant CAT activity in transiently transfected SV40-transformed human keratinocytes (SVHK cells), that were further stimulated by 12-O-tetradecanoylphorbol-13-acetate (TPA), a potent protein kinase C activator. Sequence analysis of the gene detected three TPA responsive elements (TRE-1, TRE-2, and TRE-3) and one AP-2 site on the 5′ upstream promoter region. Deletion analyses of the p2672CAT vector demonstrated that TRE-2, which was located between −272 and −278, was critical for the regulation by TPA. Gel shift analyses revealed that c-Jun, JunD, and c-Fos bound to the TRE-2 region and that the p2672CAT activity level was elevated by co-transfection with c-Jun and c-Fos or with JunD and c-Fos expression vectors. Furthermore, co-transfection of SVHK cells with the protein kinase C-α expression vector and the p2672CAT expression vector also resulted in an increased CAT activity. These results indicate that the 5′-flanking region of the human cystatin A gene confers promoter activity and contains a TRE (TRE-2) that mediates, at least in part, the enhanced expression of this gene by TPA.

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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) AB007773 and AB007774.

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Cytochalasin A is a cysteine proteinase inhibitor that belongs to family 1 of the cystatin superfamily. Cystatin A was originally isolated from polymorphonuclear granulocytes (1), but it has also been isolated from the spleen, liver, and epidermis (2–4). The primary structure of cystatin A consists of a polypeptide chain of 98 amino acid residues that is mainly distributed intracellularly (5). We have recently reported that cystatin A is identical to keratolin, one of the precursor proteins of cornified cell envelope (CE)5 (6), which is formed during terminal differentiation of keratinocytes (7–9).

CE is a highly insoluble structure formed beneath the plasma membrane of keratinocytes during terminal differentiation (7–9). This structure is 15–20 nm thick and is stabilized by cross-link formation of precursor proteins by N-(γ-glutamyl)lysine isodipeptide bonds and disulfide bonds, which are catalyzed by transglutaminase(s) and sulphydryl oxidase, respectively (7, 8, 10).

In addition to cystatin A, several proteins have been implicated as precursors of CE, which include involucrin (11), loricrin (12), small proline-rich protein(s) (13), elafin (14), and envolakin (15). Recent evidence suggests that involucrin is an early component of CE and provides a scaffold for the incorporation of other precursor proteins (14, 16).

TPA, which is a potent activator of protein kinase C (PKC), induces terminal differentiation of keratinocytes (17, 18). Recent studies have revealed that involucrin, loricrin, and transglutaminase 1 genes contain a TRE(s) in their 5′-flanking regions and that these TREs induce increased expression of these protein transcripts by TPA (19–23). We have previously shown that the mRNA level of cystatin A is also stimulated by TPA in SV40-transformed human keratinocytes (SVHK cells) (6).

SVHK cells are a well-established, immortalized cell line sharing features of normal human keratinocytes (24, 25). These cells express relatively high levels of cystatin A as compared with other cell lines, such as A432 and SCC13 (data not shown). In the present study, we have identified the structure of the human cystatin A gene by screening a human genomic library and by using the polymerase chain reaction (PCR). We have also analyzed the regulation of cystatin A promoter activity by using a CAT reporter vector, which was connected to the 5′-flanking region of the cystatin A gene.

EXPERIMENTAL PROCEDURES

Cell Culture—SV40-transformed human keratinocytes (SVHK cells) (24) were a generous gift from Dr. M. L. Steinberg (Department of
Chemistry, City College of the City University of New York, NY. The cell line was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and incubated at 37 °C with 5% CO2. 

Screening of the Human Genomic DNA Library—A human phage library was purchased from CLONTECH (Palo Alto, CA). A 448-bp human cystatin A cDNA was digested with EcoRI (6) and labeled with [32P]dCTP by the random priming method. Filter hybridization was used to screen 1 × 106 clones with a probe at 65 °C overnight in a solution composed of 1 × NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.1% SDS, and 5 × Denhardt’s solution (1× Denhardt’s solution: 0.002% polyvinyl alcohol, 0.002% Ficoll, and 0.002% bovine serum albumin). Subsequently, the filters were washed three times for 10 min at room temperature with 2 × SSC (1× SSC: 0.15 μM NaCl, 0.0015 μM sodium citrate), 0.05% SDS and twice for 1 h at 65 °C with 1× SSC, 0.1% SDS. The filters were then exposed to Kodak XAR film at −70 °C for 2 days.

PCR Cloning Strategies—PCR was performed to analyze the promoter, the first exon, and the first intron of cystatin A using the Promoter Finder DNA Walking Kit (CLONTECH, Palo Alto, CA). To isolate the promoter and the first exon, we performed the first long PCR with the AP1 primer (as described in the kit) and the HCA1 primer (5′-AGTTGGCGGTGTTGGCTCAGATAAGCCTGG-3′; +63 to +95). Subsequently, the second PCR was performed with the AP2 primer (as described in the kit) and the HCA2 primer (5′-AGTAAAGCTGCGGTAAGATTGTAGAAGCTGGAGGC-3′; +51 to +77). To isolate the first exon and the first intron, we performed the first long PCR with the AP3 primer (5′-GCCCTAGATGTCATCTAATAAGG-3′) and the HCA3 primer (5′-GC-GCAAATGCTAGCTAGCTAGCAGT-3′; +63 to +95). The second PCR was performed with the AP4 primer (5′-AACCGCGGCTCCCTAG-3′) and the HCA4 primer (5′-GGAGGATCTGCTGGAAGCACCAGCCTC-3′; +66 to +95). The HCA 1–4 primers were determined by cDNA analysis of keratolinin (6). The long PCR kit was purchased from Takara Shuzo Co. (Otsu, Japan). The DNA was amplified for 35 cycles on a DNA cyclers (Perkin-Elmer Corp., Norwalk, CT) at 98 °C for 20 s and then 68 °C for 15 min. The PCR products (pTA648, pTA2672, and pTA4.0) were subcloned into the pCR™ 2.1 vector (Invitrogen, San Diego, CA).

DNA Sequencing—The isolated clone (pHCA) was digested with EcoRI and ligated into the pGEM-3Zf(+) vector. The constructed plasmids were denatured with 0.2 μl sodium hydroxide. The single-stranded DNA was sequenced by the dideoxy chain termination method using the SP6 promoter, the first exon, and the first intron of cystatin A using the T7 promoter primers (26).

Plasmid Constructs—The HindIII, XbaI-digested fragments from the pTA2672 and pTA648 vectors were inserted into the promoterless pTA2672 and pTA648 vectors, respectively. Deletion vectors p478CAT, p238CAT, and p68CAT were generated by PCR using oligonucleotide primers coding the first and the second exon (data not shown). Between the two deletions, fragments were generated (29). The enzyme activity level of β-galactosidase in the transfected cell extracts was measured spectrophotometrically (26). Relative CAT activity is expressed as the count of acetylated fraction corrected for the activity of the 0-CAT vector.

Nuclear Extraction and Gel Retardation Analyses—Nuclear extraction and gel retardation analyses were performed as described previously (30). The oligonucleotide probe that was used corresponds to the 240 to 266 fragment, which includes the TRE-2 site (see under “Results and Discussion”).

Materials—Dulbecco’s modified Eagle’s medium was purchased from Life Technologies, Inc. Penicillin and streptomycin were obtained from M. A. Bioproducts (Walkersville, MD). The pGEM3zf(+) vector was purchased from Promega (Madison, WI). The [α-32P]dCTP and deoxy-cytidine thiophosphate (1000 Ci/ml) were purchased from Amersham Pharmacia Biotech. All other chemicals were obtained from Nakarai Chemicals Ltd. (Kyoto, Japan). Anti-c-Jun, anti-Jun B, anti-Jun D, anti-c-Fos, anti-NF-κB, and anti-Fra-1 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

RESULTS AND DISCUSSION

Identification and Structure of the Human Cystatin A Gene—A human genomic phage library was screened with a 32P-labeled, full-length human cystatin A cDNA (6). Of the 1 × 106 clones screened, one clone (pHCA) was identified that intensely hybridized with the cystatin A cDNA probe. DNA sequence analysis revealed that pHCA contained the second and the third exon of the cystatin A gene (Figs. 1 and 2). In order to isolate the first exon and the promoter region, PCR was performed with the AP1/HCA1 primer pair and the AP2/ HCA2 primer pair (see “Experimental Procedures”). Two fragments were obtained, one 648 bp and the other 2672 bp. These fragments were subcloned into pCR™ 2.1 vector (pTA648 and pTA2672; see Fig. 1), and DNA sequence analysis was performed. These fragments contained the first exon with the 5′-untranslated region (Figs. 1 and 2). In order to isolate the first exon and the first intron, PCR was performed with the AP3/HCA3 primer pair and the AP4/ HCA4 primer pair. A fragment of 4 kb (pTA4k) was amplified, and DNA sequence analysis revealed that the fragment contained the first exon with a portion of the first intron. From these results, cystatin A was shown to contain three exons and two introns. The first, second, and third exons consisted of coding sequences of the cystatin A gene that were 66, 102, and 126 bp in length (Fig. 2). The second intron was 3.6 kb in length, and the first intron was 14 kb in length, as determined by PCR using oligomers coding the first and the second exon (data not shown). We have previously shown that TPA increases the level of mRNA in SVHK cells (6). Therefore, the transcription initiation site of the cystatin A gene was determined by the primer extension method using RNA prepared from TPA-treated cells.
treated SVHK cells. The transcription initiation site was located 55 bp upstream from the first translation site (Fig. 2, A).

Cysteine proteinase inhibitors have been subdivided into three families based on primary structure, molecular weight, number of disulfide bonds, and subcellular localization (5). Family 1 cystatins (cystatins A and B) consist of approximately 100 amino acid residues (11–12 kDa) and lack disulfide bonds. Family 2 cystatins (cystatins C, S, and D) are approximately 120 amino acids in length (13–14 kDa) and contain two disulfide bonds. Family 3 cystatins, also known as the kininogen family, contain nine disulfide bonds. The human cystatin A gene consists of three exons and two introns similar to cystatin B (family 1), cystatin C (family 2), cystatin S (family 2), and cystatin D (family 2) (31–34). DNA sequence analyses showed that the 5′-flanking region of the human cystatin A gene did not contain a CAAT box or a TATA box. The other cystatin genes, except for cystatin C, also do not contain these sites.

Identification of the Basal Promoter Region of the Human Cystatin A Gene—In order to determine the basal promoter region of the human cystatin A gene, six deletion fragments spanning from +77 to −2585 in the 5′-flanking region were fused with the CAT gene and transfected into SVHK cells (Fig. 3). The construct containing the +77 to −2585 fragment (p2672CAT) expressed a CAT activity level 13 times as high as the reverse-oriented construct (Fig. 3) or the constructs with vector but with no flanking region (data not shown). These data indicate that the 5′-flanking region of the human cystatin A gene contains a sequence that confers promoter activity. Deletion of the p2672CAT fragment up to −2238 demonstrated minimal loss in basal activity. When the 5′-flanking region was deleted to the −268 position, the CAT activity was markedly depressed, suggesting that the most proximal −238 bp of the 5′-flanking region is essential for the basal transcription.

Within −68 to −238, the cystatin A promoter contains an AP-2-like sequence (TCCCCATGCC; −75 to −84). AP-2 is an enhancer-binding protein that has been purified and cloned...
from HeLa cells, and this protein specifically interacts with the consensus sequence (T/C)(C/G)CC(A/C)N(GCG/CGC) (35).

Preliminary analysis showed that deletion of the AP-2 region decreased the basal promoter activity by one-third. The AP-2-like site might contribute to the basal transcription of human cystatin A gene.

The TRE-2 Site (−272 to −278) Is Critical for the Up-regulation of the Cystatin A Gene by TPA—Cystatin S, which is highly expressed in the salivary gland, is induced by the β-adrenergic agonist isoproterenol (36). Previously, we have reported that cAMP and TPA increases the mRNA level of cystatin A in SVHK cells (6). So far, there is no evidence for the TPA-dependent induction of other cystatins. To determine whether transcription of the human cystatin A gene is stimulated by TPA, five deletion constructs were transfected into SVHK cells in the presence or absence of TPA. The results showed that the construct containing the fragment +77 to −478 responded to TPA stimulation. The CAT activity level increased 3-fold following a 24-h exposure to TPA (Fig. 4). Consistent with the fact that the effect of TPA is mediated by PKC, the TPA-dependent cystatin A promoter activity was mimicked by other PKC activators, 1-oleoyl-2-acetylglycerol and mezerein (Fig. 5A). 4-O-methyl-phorbol 12-myristate 13-acetate, a very weak PKC activator, produced much less effect on the promoter activity. Furthermore, the effect of TPA was inhibited by the PKC inhibitor 1-(5-isquinoline-sulfonyl)-2-methyl piperazine dihydrochloride (Fig. 5B).

There are two putative TPA responsive elements (TRE-1, −189 to −195; TRE-2, −272 to −278) and one AP-2 responsive site (−74 to −83) within the +77 to −478 region of the cystatin A gene (Fig. 2). In order to determine the critical region of the TPA regulatory site, three TRE-deleted constructs were transfected into SVHK cells. Deletion of the T-2 region (−272 to −278) or the T-2 plus T-1 region (−189 to −195) completely abolished the TPA responsiveness (Fig. 6). Conversely, deletion of T-1 showed no significant loss in TPA responsiveness. These results indicate that the sequence −272 to −278 (TRE-2) is responsible for the TPA stimulation.

In keratinocytes, TPA is a potent inducer of differentiation and increases the expression of CE precursor protein(s), as well as transglutaminase. TRE sites have been identified in a number of differentiation-related genes, such as loricrin, involucrin, small proline-rich protein(s), and transglutaminase 1 (19–23). Our study revealed that the TRE-2 region (−272 to −278) of the cystatin A gene was critical for the TPA-induced promoter activity. This is consistent with a common controlling mechanism for the expression of CE precursor proteins, as well as their cross-linking enzyme, transglutaminase 1.

c-Jun, JunD, and c-Fos Bind to the TRE-2 Region and Increase the Cystatin A Promoter Activity—The AP-1 protein, which is a complex consisting of Jun and Fos family proteins, binds to TREs and regulates the TPA-inducible genes. In order to determine the binding protein(s) in the TRE-2 region of the human cystatin A gene, a 38-bp synthetic oligonucleotide representing the TRE-2 region (−272 to −278 bp) was evaluated using a DNA gel shift assay. Incubation of the oligonucleotide with the nuclear extract from TPA-treated SVHK cells yielded three DNA-protein binding complexes (Fig. 7, lane 2). The specificity of the binding was verified by a competition assay using the same (Fig. 7, lane 4) or unrelated (Fig. 7, lane 5) unlabeled oligonucleotides in excess of 100 moles. Furthermore, anti-c-Jun, anti-JunD, and anti-c-Fos antibodies decreased the specific bands, whereas supershifted bands appeared near the top of the lane (Fig. 7, lanes 6, 8, and 9). A supershifted band was not detected by the addition of anti-JunB, anti-Fra-1, or anti-NF-kB antibodies (Fig. 7, lanes 7, 10, and 11).

In order to determine the effects of these AP-1 related proteins on the transcription of cystatin A, various expression vectors of the Jun and Fos family proteins were transfected into SVHK cells. Co-transfection of the p648CAT vector with the c-Jun and c-Fos expression vectors or the JunD and c-Fos expression vectors resulted in an increase in the CAT activity level (Fig. 8). This finding suggests that the nuclear proteins that bind to the TRE-2 region are most likely composed of c-Jun and c-Fos or of JunD and c-Fos.

It has been reported that cystatin A is expressed in the upper spinous layer to the granular layer of the normal epidermis.
Various TRE-deleted p648CAT vectors were transfected into SVHK cells, and the cells were treated with TPA (10 ng/ml) for 24 h. The nuclear extracts from TPA-treated SVHK cells were reacted with the synthesized oligomer containing the TRE consensus sequence; lane 2, nuclear extract from TPA-treated SVHK cells with the anti-c-Jun antibody; lane 3, nuclear extract from TPA-treated SVHK cells with the anti-c-Fos antibody; lane 4, nuclear extract from TPA-treated SVHK cells with the anti-JunD antibody; lane 5, nuclear extract from TPA-treated SVHK cells with the anti-Fra-1 antibody; lane 6, nuclear extract from TPA-treated SVHK cells with the anti-NF-kB antibody; lane 7, nuclear extract from TPA-treated SVHK cells plus the oligomer containing the TRE consensus sequence; lane 8, nuclear extract from TPA-treated SVHK cells with an excess of 100 moles of unlabeled probe; lane 9, nuclear extract from TPA-treated SVHK cells with the anti-c-Jun antibody; lane 10, nuclear extract from TPA-treated SVHK cells with the anti-c-Fos antibody; lane 11, nuclear extract from TPA-treated SVHK cells with the anti-c-Jun antibody; lane 12, nuclear extract from TPA-treated SVHK cells with the anti-NF-kB antibody. The arrows indicate the supershifted bands.

Transcription of the Cystatin A Gene Is Increased by Transfection of the PKC-α Expression Vector—PKC is a large family of proteins consisting of at least 11 isoforms (39). PKC-α, βII, βΙΙΙ, and γ are the classical PKC proteins that are calcium- and diacylglycerol-dependent. PKC-δ, ε, η, θ, and ι-ζ are the novel PKCs, which do not require calcium for activation. PKC-ζ and -η are the atypical PKCs, which require neither calcium nor diacylglycerol for activation. TPA activates classical and novel PKCs but not atypical PKCs. The epidermal keratinocytes contain PKC-α, δ-ε, η, θ, and ζ (40).

In order to determine the PKC isozyme(s) responsible for cystatin A gene expression, we co-transfected SVHK cells with the p648CAT vector and various PKC isozyme expression vectors. Consistent with TPA-induced activation of endogenous PKC(s), cystatin A promoter activity was increased by TPA in SVHK cells transfected with the control vector (Fig. 9, C). Transfection of PKC-δ, ε, η, or ζ had no effect on the cystatin A promoter activity as compared with the transfection of the control vector. Although TPA also increased the promoter activity of SVHK cells transfected with PKC-δ, ε, η, or ζ, the increase was not statistically significant as compared with that of the cells transfected with the control vector (Fig. 9, δ, ε, η, γ, and ζ). Cystatin A promoter activity, however, was significantly stimulated by co-transfection of p648CAT and the PKC-α vectors, which was further stimulated by TPA (Fig. 9, α). These results suggest that PKC-α is responsible for the stimulation of the human cystatin A promoter activity. There are several reports concerning the localization of PKC isoforms in the epidermis (41–43). In normal skin, PKC-η is expressed in the uppermost granular layer (43), whereas PKC-α mRNA is expressed from the basal to the spinous layers (42). Because cystatin A is expressed in the upper spinous layer and the granular layer, there would be other transcription factors (either stimulatory or inhibitory) that regulate the differentiation-specific expression of cystatin A in the epidermis.

In this study, we characterized the structure of the human...
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