Cloning and Expression Analysis of a Novel Salicylate Suppressible Gene, *Hs-CUL-3*, a Member of Cullin/Cdc53 Family*

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By using a mRNA differential display technique to search for salicylate suppressible genes, we identified a cDNA in human foreskin fibroblasts, which by GenBank™ DNA data base search shows sequence homology to the recently reported cullin/Cdc53 (CUL) family genes, especially CUL-3. We have cloned the full-length human CUL-3 (*Hs-CUL-3*) cDNA. It encodes a 768-amino acid polypeptide and has a predicted molecular weight of 88,939. The amino acid sequence of *Hs-CUL-3* shows 46% homology to that of its Caenorhabditis elegans ortholog, Ce-CUL-3, and 27 and 23% to that of *Hs-CUL-1* and *Hs-CUL-2*, respectively. Northern blot analysis showed that phorbol 12-myristate 13-acetate increased the expression of *Hs-CUL-3* mRNA in a concentration- and time-dependent manner, and this increase was inhibited by sodium salicylate. *Hs-CUL-3* widely expressed in human tissues and its expression in cultured COLO205 colon cancer cells was increased when compared with that in normal colon cells. It is likely that *Hs-CUL-3* is involved in cell proliferation control.

Nonsteroidal anti-inflammatory drugs contain widely prescribed agents, including aspirin and salicylic acid. It is well documented that aspirin exerts its anti-inflammatory action by inhibiting the activity of cyclooxygenase (COX), 1 which is a key enzyme in catalyzing the biosynthesis of prostaglandins (1, 2). Recent studies indicate that the inducible isoform, COX-2, plays a key role in inflammation (3, 4). COX-2 induction has been implicated in colon cancer proliferation (5, 6). Aspirin has weak and nonselective anti-COX action, whereas salicylate is inactive against COX-2, suggesting that the anti-inflammatory action of aspirin and salicylate may be mediated by a mechanism other than inhibition of COX activity. We postulate that salicylate may suppress certain inducible genes that are important in inflammation and tumor cell proliferation. To identify and isolate new inducible genes whose expression is suppressed by salicylate, we performed mRNA differential display utilizing human fibroblasts that were untreated, treated with phorbol 12-myristate 13-acetate (PMA), or treated with PMA and salicylate. A series of genes were identified. In this report we describe the isolation of a full-length cDNA that has sequence homology with genes in the cullin/Cdc53 (CUL) family (7). Searching the GenBank™ DNA data base reveals that our cDNA sequence matches the reported partial sequence of human CUL-3 (7). Our results show that *Hs-CUL-3* is widely distributed in human tissues. Its expression in human fibroblasts is increased by PMA, and this increase is suppressed by salicylate. Furthermore, its expression is increased in cultured COLO205 colon cancer cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatment**—Human foreskin fibroblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) and a 1:100 dilution of an antibiotic-antimycotic solution. When reaching near-confluence, the cells were cultured in the Dulbecco’s modified Eagle’s medium supplemented with 0.5% FBS for 22–24 h and then treated with 100 nM PMA for 3 h before harvest. Sodium salicylate, which was dissolved in culture medium at 1 mM concentration, was added to the medium at the final concentration of 1 or 10 mM 30 min before PMA treatment. Human colon CCD-33Co cells were cultured in minimum essential medium supplemented with non-essential amino acids, 10% FBS, and antibiotic-antimycotic solution. Human colon adenocarcinoma COLO205 cells were cultured in RPMI medium 1640 supplemented with 10% FBS and antibiotic-antimycotic solution. All the tissue culture reagents were obtained from Life Technologies, Inc.

**mRNA Differential Display**—mRNA differential display was done as described by Liang and Pardee (8) using an RNAmap kit (GenHunter). Briefly, total RNA pretreated with DNase I using a MessageClean kit (GenHunter) was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase and an anchored oligo-d(T) primer, followed by the PCR reaction with the same oligo-d(T) primer and a second arbitrary primer. PCR was performed under the following conditions: 94°C, 30 s; 40°C, 2 min; 72°C, 30 s for 40 cycles followed by 72°C for 5 min, in the presence of [32P]dATP. An equal amount of PCR products from each reaction was electrophoresed on a 6% denaturing polyacrylamide gel. The gel was dried on 3M paper and exposed to Kodak X-OMAT MR film overnight. Bands of interest were excised and eluted from the gel, realigned with the same primer set, and cloned into pGEM-T vector (Promega).

**5'-RACE**—To obtain full-length cDNA sequence of *Hs-CUL-3*, the 5'-RACE approach was used. First, cDNA was synthesized from mRNA isolated from PMA-treated fibroblasts. Adaptor ligation and PCR were performed by using a marathon cDNA amplification kit according to the manufacturer’s recommendations (CLONTECH).

**DNA Sequencing and Sequence Analysis**—Plasmids containing full-length cDNA sequences were sequenced by chain-termination DNA sequencing method with T7 Sequenase version 2.0 DNA polymerase (Amersham Pharmacia Biotech). GenBank™ data base was used for nucleotide sequence search and Lasergene (DNAstar, Inc.) for sequence analysis and alignment.

**Northern Blot Analysis**—The procedure was described previously (9). Total cellular RNA (5 or 10 μg) was applied to and run on 1% denaturing formaldehyde-agarose gels and transferred onto positively charged
nylon membrane. Filters were hybridized with \[32P\]dCTP-labeled full-length Hs-CUL-3 cDNA and, after stripping, rehybridized with \[32P\]dUTP-labeled GAPDH RNA probe as control. Premade human multiple tissue Northern blot was purchased from CLONTECH (number 7760-1). According to the manufacturer's information, each lane was loaded with 2 mg of poly(A)\(^1\)RNA prepared from whole heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas tissues. The premade blot was hybridized with full-length Hs-CUL-3 cDNA probe and, after stripping, rehybridized with human \(\beta\)-actin cDNA probe as control.

**RESULTS**

Of a number of cDNA fragments identified from the differential display sequencing gels, one was of particular interest because a search of the GenBank\(^7\) revealed that the sequence of this 408-bp fragment was homologous to the recently identified CUL multigene family, especially CUL-3 (7). Although the 3013-bp \textit{Caenorhabditis elegans} CUL-3 (\textit{Ce-CUL-3}) appears to be a full-length cDNA encoding a 780-amino acid polypeptide, the human CUL-3 cDNA (\textit{Hs-CUL-3}) sequence in the expressed sequence tag data base is only 2092 bp in length, which lacks the 5'-coding sequence (7). We used 5'-RACE with a \textit{Hs-CUL-3}-specific oligonucleotide primer and amplified a 757-bp fragment at the 5'-region. Sequencing results showed that this fragment overlapped with the reported 2092-bp sequence of \textit{Hs-CUL-3} cDNA and contained an additional 5'-coding region, extending the sequence from 2092 to 2653 bp. A search of the expressed sequence tag data base with the extended 5'-end sequence identified a 484-bp 5'-end cDNA sequence, further adding 83 bp to the 5'-end of the \textit{Hs-CUL-3} cDNA sequence. The overall cDNA sequence of \textit{Hs-CUL-3} is 2746 bp (Fig. 1). It contains a single open reading frame that encodes a putative protein of 768 amino acid residues. The sequence (ACCATGT) containing the putative translation initiation site complies with Kozak's rule (10, 11), supporting that the \textit{Hs-CUL-3} cDNA we cloned contained a complete coding region. Similar consensus sequences were also found in other members of CUL family. \textit{Hs-CUL-3} is a basic protein with a predicted molecular weight of 88,939 kDa. The amino acid sequence homology between Hs-CUL-3 and Ce-CUL-3 is 46%, whereas the similarity of Hs-CUL-3 to other CUL proteins is 17–27%. The C-terminal region of all CUL proteins is highly conserved (Fig. 2).

Northern blot analysis of human fibroblasts with or without PMA treatment revealed that \textit{Hs-CUL-3} expressed constitutively in untreated cells, and PMA increased its expression level by about 2-fold in a time- and concentration-dependent manner with the maximal induction by 100 nM PMA at 4 h (Fig. 3, A and B). It is notable that two \textit{CUL-3} transcripts were detected in all of these Northern blots with a major band of about 2.8 kb and a minor band of about 4.3 kb. This induction was inhibited by salicylate at 1–10 mM (Fig. 3C). These results corroborated the \textit{Hs-CUL-3} expression pattern in mRNA differential display. As shown in Fig. 3D, only PMA caused an
increase in CUL-3 mRNA expression. Interleukin-1β, tumor necrosis factor-α, or lipopolysaccharide had no effect. The Hs-CUL-3 mRNA level was increased in COLO205 colon cancer cells when compared with normal colon cells (Fig. 4A). These results suggest that CUL-3 may be involved in cell proliferation. Both the major 2.8-kb and the minor 4.3-kb bands of CUL-3 transcripts were detected in parallel in human tissues examined with different intensities (Fig. 4B). The highest expression level was observed in skeletal muscle and heart tissues. A relatively high expression level was also found in placentas. In liver and lung, Hs-CUL-3 transcripts were scarcely detected. The β-actin mRNA level was also highest in heart and skeletal muscle tissues as reported previously.

**DISCUSSION**

Cullins/Cdc53 are a recently identified family of proteins with five known members in *C. elegans* (six in *Homo sapiens* and three in *Saccharomyces cerevisiae* (7)). In this study, we report for the first time isolation of the full-length Hs-CUL-3 cDNA, which shares with other members of human CUL proteins only between 20 and 35% of the overall amino acid sequence identity. The function of CUL proteins remains to be ascertained. In *C. elegans*, mutation of CUL-1 causes hyperplasia of all tissues, leading to the suggestion that it is a required element for developmentally programmed cell cycle exit from G1 to G0 (7). In yeast, Cdc53 was reported to target phosphorylated G1 cyclins for degradation by the ubiquitin proteolytic pathway (12, 13). Cdc53 forms a ubiquitin ligase complex named SCFCdc4 with Skp1 and Cdc4 to catalyze ubiquitin-dependent phosphorylation and degradation of Sic1, an S-phase cyclin-dependent kinase inhibitor (14, 15). The inactivation of Sic1 is required for G1 to S phase transition (16). Selective recognition of phosphorylated Sic1 is controlled by Cdc4, and the interaction between Cdc4 and Sic1 is enhanced by Skp1. Cdc53 is thought to function as an adapter linking Skp1/Cdc4 to Cdc34. A similar model has been proposed for a human protein complex consisting of CUL-1, p19^{Skp1}, and p45^{Skp2}, a protein required for S phase in human (17). Despite a low degree of overall sequence identity with other members of CUL...
family, Hs-CUL-3 shares with them a high level of homology at several regions, especially at the C-terminal region, suggesting a closely related structure. It is likely that Hs-CUL-3, like CUL-1 and Cdc53, may be involved in regulating cell cycle progression.

Given that each component of the proposed yeast ubiquitin ligase complex SCF\(^\text{Cdc4}\), i.e. Cdc4, Cdc53, and Skp1, belongs to a distinct protein family, the proposed SCF\(^\text{Cdc4}\) model may represent a prototype for a variety of ubiquitin ligase complex E3s. This is supported by a recent report that degradation of G1 cyclin Cln2 in yeast appears to require a ubiquitin ligase complex consisting of Cdc53, Skp1, and Grr1, instead of Cdc4 (12, 18). Both Cdc4 and Grr1 contain a conserved sequence, F-box, and several regions, especially at the C-terminal region, suggesting a closely related structure. It is likely that Hs-CUL-3, like CUL-1 and Cdc53, may be involved in regulating cell cycle progression.

For the 2.8-kb Hs-CUL-3 transcript was normalized to GAPDH transcript levels and expressed relative to control. Hs-CUL-3 was expressed relative to control and reproduced for \(\beta\)-actin as control.

Phosphorylation and degradation by the ubiquitin proteolytic pathways are considered to be a common mechanism for controlling many regulatory proteins. Diverse SCF-like ubiquitin-dependent proteolytic pathways may be present in eukaryotic cells (14), which may play a broad role in regulating biological processes such as cell proliferation (15). In this study we found that Hs-CUL-3 mRNA levels are stimulated by PMA in human fibroblasts and are increased in COLO205 colon cancer cells. These results suggest that CUL-3 is involved in cell proliferation through its participation in SCF complex formation. It is interesting to note that salicylate suppresses CUL-3 mRNA accumulation by PMA. Sodium salicylate and aspirin have been shown to suppress gene expressions via inhibition of I\(\kappa\)B phosphorylation and degradation and consequently blocking NF-\(\kappa\)B activation (24–26). It would be interesting to study whether CUL-3 is involved in I\(\kappa\)B degradation through the ubiquitin pathway.

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