Expression of Human Cystatin A by Keratinocytes Is Positively Regulated via the Ras/MEKK1/MKK7/JNK Signal Transduction Pathway but Negatively Regulated via the Ras/Raf-1/MEK1/ERK Pathway

Cystatin A, a cysteine proteinase inhibitor, is a cornified cell envelope constituent expressed in the upper epidermis. We previously reported that a potent protein kinase C activator, 12-O-tetradecanoylphorbol-13-acetate, increases human cystatin A expression by the activation of AP-1 proteins. Here, we delineate the signaling cascade responsible for this regulation. Co-transfection of the cystatin A promoter into normal human keratinocytes together with a dominant active form of ras increased the promoter activity by 3-fold. In contrast, a dominant negative form of ras suppressed basal cystatin A promoter activity. Further analyses disclosed that transfection of dominant negative forms of raf-1, MEK1, ERK1, ERK2, or wild-type MEKK1 all increased cystatin A promoter activity in normal human keratinocytes, whereas wild-type raf-1, ERK1, ERK2, or dominant negative forms of MEKK1, MKK7, or JNK1 suppressed the promoter activity. The increased or decreased promoter activity reflected the expression of cystatin A on mRNA and protein levels. These effects were not observed when a cystatin A promoter with a T2 (−272 to −278) deletion was used. In contrast, transfection of dominant negative forms of MKK3, MKK4, or p38 did not affect cystatin A promoter activity. Immunohistochemical analyses revealed that phosphorylated active extracellular signal-regulated kinases and c-Jun N-terminal kinase were expressed in the nuclei of basal cells and cells in the suprabasal-granular cell layer, respectively. These results indicate that the expression of cystatin A is regulated via mitogen-activated protein kinase pathways positively by Ras/MEKK1/MKK7/JNK and negatively by Ras/Raf/MEK1/ERK.

During their migration from the basal cell layer to the horny cell layer of the skin, keratinocytes cease proliferation. They then undergo terminal differentiation, which is characterized by the production of a highly insoluble rigid structure termed cornified cell envelope (CE) beneath the plasma membrane (1–3). Transglutaminase enzymes catalyze the assembly of this structure via formation of ε-γ-glutamyllysine bonds between envelope precursors. The proteins that have been identified as constituents of the CE include involucrin (4), loricrin (5), small proline-rich protein(s) (7), elafin (8), envoplakin (9), desmosomal components (10), and plasminogen-activator inhibitor 2 (10). Recent evidence indicates that involucrin is an early component of CE and provides a scaffold onto which other precursor proteins are incorporated (8, 10).

Cystatin A, a cysteine proteinase inhibitor, belongs to the cystatin superfamily. In the epidermis cystatin A is expressed in the upper spinous to granular cell layers (12). In addition to its proteinase inhibitory effect, cystatin A is known to be an early precursor protein of CE in keratinocytes (8).

The human cystatin A gene is located on 3q21 (13) and consists of three exons that are separated by 14- and 3.6-kilobase introns, respectively (14). A potent protein kinase C activator, 12-O-tetradecanoylphorbol-13-acetate (TPA), induces terminal differentiation of keratinocytes (15, 16). Recent studies revealed that TPA increases cystatin A expression at both the mRNA and protein levels (6). The 5′-flanking region of the cystatin A gene contains at least two putative TPA-responsive regions, T1 (−189 to −196) and T2 (−272 to −278). The AP-1 proteins, c-Jun, c-Fos, and Jun D, regulate cystatin A promoter activity via PKC activation (8). However, the precise regulatory mechanism of AP-1-dependent cystatin A expression in keratinocytes remains to be determined.

Cell growth, differentiation, and apoptosis are mediated by the activation of mitogen-activated protein kinase (MAPK) pathways. A MAPK is activated by a specific MAPK kinase (MAPKK) through the phosphorylation of specific threonine and tyrosine residues (Thr-X-Tyr) in MAPK. MAPKK is activated by MAPKK kinase through the phosphorylation of specific threonine and tyrosine residues (Thr-Y-Tyr) in MAPKK (17, 18). These kinases constitute MAP kinase cascades. In mammalian cells, at least four MAPKs, namely, extracellular signal-regulated kinases (ERKs) (19), c-Jun N-terminal kinase/stress-activated protein kinases (JNKs/Apoptosis signal-regulated kinases (JNKs)) (20), p38 (21), and ERK5/big MAP kinase have been identified (22). Although

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1 The abbreviations used are: CE, cornified cell envelope; TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; NHK cell, normal human keratinocyte; CAT, chloramphenicol acetyltransferase; wt, wild type; dn, dominant negative; da, dominant active.
the ERKs are usually activated by mitogenic stimuli. JNK and p38 are activated by other stimuli such as environmental stress, ultraviolet irradiation, osmotic pressure, and various cytokines including tumor necrosis factor α and transforming growth factor β (23, 24). ERK/big MAP kinase 5 seems to play a significant role in epidermal growth factor-induced cell proliferation (22). Recent analyses indicate that TPA-induced gene expression of keratinocytes is associated with MAP kinase activation. For example, the expression of the human involucrin gene is regulated by MAPK cascades (25). In the present study, we have analyzed the regulatory pathway(s) of various MAPK-signaling systems that control cystatin A expression of normal human keratinocytes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Normal human keratinocytes (NHK cells) were obtained during plastic surgery. Informed consent was obtained from the patients. The cells were cultured in keratinocyte growth medium containing epidermal growth factor (10 ng/ml), insulin (5 μg/ml), and bovine pituitary extract (50 μg/ml) at 37°C in 5% CO2 in air. NHK cells were maintained in a subconfluent state by subculturing every 4–5 days. Cells were seeded into 60-mm diameter plastic dishes at concentrations of 1–2 × 10^5 cells/ml.

**Plasmid Constructs**—We previously have published the structure of the cystatin A promoter construct (17–648) linked to the chloramphenicol acetyltransferase (CAT) gene (pΔ48CAT) (14). The structure of pΔ48ΔT1 and pΔ48ΔT2, with the deleted TPA-responsive elements T1 (189–196) and T2 (272–278), respectively, has been described previously (14).

**Transfection and CAT Assay**—The transfection of plasmid DNA into cells was performed by the liposome method using Lipofectin (34). Typically, 5 μg of reporter plasmid and 2 μg of β-galactosidase plasmid were co-transfected in 2 μg of various expression vectors or empty vector into 1 × 10^5 NHK cells. The β-galactosidase plasmid was used as the internal standard to normalize the efficiency of each transfection. After 48 h, the cells were collected, and CAT assays were performed (35). The enzyme activity of β-galactosidase in the transfected cell extracts was measured spectrophotometrically (36). Relative CAT activities were expressed as the fold increase in the acetylated fraction after correction for the activity of the 0-CAT vector.

**Construction of Adenovirus Vectors and Transfection into NHK Cells**—The cDNA of dominant negative mutants of adenoviral regulatory (dn-ERK1, human REK1 K52R) and dn-ERK2 (rat ERK2 K71R) were provided by Dr. Melanie Cobb (26). Dominant negative Ha-ras (S17V) (dn-ras) and dn-MEK1, cloned in pRSa, and wt-Raf-1 and dn-raf-1 (K375W), each cloned in pRSV, were kindly provided by Dr. Michael Karin (27). Constitutively active ras (G12V) (dn-ras) and constitutive MEK1, and dn-MEK4 (S220A), each cloned in pEECMV, were provided kindly by Dr. Dennis Templeton (29, 30). Dominant negative MKK7 (Mkk7k7k) cloned in pRSa (dn-MKK7) was a kind gift of Dr. Eiuke Nishida (31). Dominant negative M KK3 (Mkk3k3a) cloned in pRSV (m-mkk3), dominant negative p38 MAPK (p38AGF) cloned in pCMV3 (m-p38), and dominant negative JNK1 (jnk1APF) cloned in pDNA3 (m-jnk) were kindly provided by Dr. Roger Davis (20, 32, 33). The β-galactosidase expression vector was provided kindly by Dr. Takeshi Watanabe (Medical Institute of Bioregulation, Kyushu University, Japan).

**Immunohistochemical Analyses**—Tissue specimens were fixed in 10% formalin, embedded in paraffin, and cut into consecutive sections of 5-μm thickness. The sections were treated with 0.3% hydrogen peroxide to block endogenous peroxidase activity. After incubation with normal goat serum diluted in PBS for 10 min, the sections were incubated for 4 h at room temperature with anti-ERK, anti-phospho-ERK, anti-JNK, or anti-phospho-JNK (New England Biolabs) antibodies diluted 1:200 in PBS. The sections were then washed with PBS followed by staining with the Histofine streptavidin-biotin (SAB)-PO(M) kit (Nichirei, Tokyo, Japan). Biotinylated goat anti-rabbit antibody was used as the secondary antibody, and the immune reaction was visualized by avidin/biotin complexing with 0.03% hydrogen peroxide as a substrate and diaminobenzidine as a chromogen. The sections were then counterstained with hematoxylin and mounted in PermaFlurTM aqueous mounting medium (IMMUNON, Pittsburgh, PA).

**RESULTS AND DISCUSSION**

**Activated Ras Induces Increased Cystatin A Promoter Activity**—Ras is a GTP-binding protein located upstream of the MAP kinase cascade (17, 18). Although the activation of Ras is usually associated with receptor tyrosine kinase activation, recent studies demonstrated that Ras is also a downstream target of PKC (44, 45). To determine whether Ras is involved in TPA-stimulated cystatin A promoter activity, the promoter vector forming assays in HEK293 cells. AxCADA/MEK, representing constitutively active human MEK1 (termed dominant active MEK1 or da-MEK1), without the nuclear export signal was constructed as described elsewhere (37). The AxCANLaCZ was provided kindly by Dr. Izumi Saito (University of Tokyo) (41). The cells were infected by adding adenovirus (multiple plaque hybrid, 10) to keratinocyte growth medium. The cells were incubated at 37°C for 60 min. The medium was changed to fresh keratinocyte growth medium, and the cells were incubated at 37°C for 24 h.

**Northern Blot Analyses**—NHK cells (5 × 10^6) were homogenized using the 6 M guanidine-cesium-guanidine method (36). Total RNA (30 μg) was electrophoresed in 1% agarose-formaldehyde gels and transferred to nylon membranes. Human cystatin A was amplified using specific oligonucleotide primers (5'-ATGATACTGAGGCTTCTATCT-3' and 5'-CACGAGGTTTCCTCGGTGAG-3'). The blot was hybridized with a 20-mer oligonucleotide (GAPDH) were labeled with [32P]dCTP by the random primer method. Hybridization was performed as described previously (36). GAPDH cDNA was a generous gift of Dr. Michael Tainsky (M.D. Anderson Cancer Center, University of Texas, Houston, TX).
(p648CAT) was transfected into NHK cells together with either da-Ras or dn-Ras vectors. As reported previously (6, 14), TPA significantly stimulated cystatin A promoter activity (Fig. 1). The stimulatory effect of da-Ras was not affected by the presence or absence of TPA (Fig. 1), suggesting that the effect of the latter on cystatin A promoter activity is mediated via the activation of Ras. In contrast, co-transfection of dn-Ras decreased both basal and TPA-stimulated promoter activity. This is consistent with the idea that TPA mediates its effects through the activation of Ras and also suggests that Ras is partially activated in NHK cells under the conditions employed (i.e. with 0.01 mM calcium).

Cystatin A Expression Is Inhibited via Raf-1-MEK1-ERK Pathway—Ras activates various MAPK cascades including Raf-1/MEK1/ERK and MEKK1/MKK3/4/6/p38/JNK path-
of dominant negative forms of Raf-1 increased basal cystatin A promoter activity (Fig. 2). On the other hand, transfection with wt-Raf-1 markedly stimulated basal cystatin A promoter activity. The stimulatory effect was similar to that of transfecting da-ras (Fig. 2). The da-ras-induced stimulation of cystatin A promoter activity was not affected by co-transfection of wt-Raf-1 or wt-ERK1/2 but was augmented by co-transfection of dominant negative forms of Raf-1, MEK1, or ERK1/2 (Fig. 2). This suggests that the Raf-1/MEK1/ERK1/2 pathway mediates the inhibitory effect on cystatin A promoter activity in NHK cells. Consistent with this, a MEK1 inhibitor, PD98059, increased cystatin A promoter activity in a dose-dependent manner (Fig. 3). This was observed even in the absence of transfection of da-Ras, again indicating that Ras is partially activated under our culture conditions. Increased promoter activity caused by PD98059 was augmented by the transfection of da-Raf-1 (Fig. 3).

Raf-1 is located upstream of ERK1. Accordingly, the stimulatory effect of dn-Raf-1 was inhibited by the co-transfection of wt-ERK1 but not by co-transfection of dn-ERK1 (Fig. 4). Co-transfection of dominant negative forms of JNK1 or p38 vectors had no effect on dn-raf-1-induced cystatin A promoter activity (Fig. 4), suggesting that the effect of dn-raf-1 is independent of JNK or p38 pathways.

These results indicate that (i) the Raf-1/MEK1/ERK pathway suppresses cystatin A expression in NHK cells, (ii) Raf-1 is functionally located upstream of MEK1 and ERK1/2 in this cascade, and (iii) the Raf-1-dependent inhibitory pathway is distinct from JNK1 or p38 pathways in NHK cells.

Cystatin A Expression Is Stimulated via MEK1/1/MKK7/JNK Pathway—In addition to the Raf-1/MEK1/ERK pathway, Ras also activates the MEK1-signaling cascade (46). MEK1 activates MKK4, which in turn activates JNK and p38 (27, 29). MEK1 also activates MKK7, which is a specific activator of JNK (31). Transfection of wt-MEK1 increased cystatin A promoter activity in NHK cells (Fig. 5). This effect was similar to that of da-ras, and co-transfection of both da-Ras and wt-MEK1 had no additive effect on the increased promoter activity (Fig. 5). This indicates that da-Ras mediates its stimulatory effect through MEKK1. Further analyses disclosed that the effect of da-Ras depends on MEK1, MKK7, and JNK but not on MKK4 or p38. The transfection of dn-MEK1, dn-MKK7, or dn-JNK suppressed basal promoter activity, whereas co-transfection of da-ras had no stimulatory effect (Fig. 5). Transfection of dn-MKK3, dn-MKK4, or dn-p38 had no effect on basal promoter activity either. Furthermore, da-Ras showed stimulatory effects in these cells, indicating that MKK3, MKK4, or p38 are involved in the Ras-dependent activation of cystatin A expression in NHK cells. Western blot analysis showed the expression of MKK3, MKK4, and p38 in NHK cells. The p38 inhibitor, SB203580, also failed to diminish the increased cystatin A promoter activity induced by wt-MEK1 (data not shown).

As described above, MEK1 is located downstream of active Ras. Consistent with this, the transfection of MEK1 stimulated cystatin A promoter activity in NHK cells (Fig. 6). Co-transfection with dn-MKK7 or dn-JNK inhibited MEK1-dependent stimulation of cystatin A promoter activity. Co-transfection of wt-ERK1 inhibited the MEK1-stimulated promoters activity, whereas dn-ERK1 and dn-MEK1 augmented it. This was expected, because the suppressive effect of the Raf-1/MEK1/ERK pathway on cystatin A expression of NHK cells was abrogated under these conditions. Co-transfection of dn-MKK3, dn-MKK4, or dn-p38 had no effect on MEK1-stimulated cystatin A promoter activity in NHK cells (Fig. 6). Western blot analysis showed the expression of MKK3, MKK4, and p38, suggesting an independent effect for promoter activity. Real-time quantitative reverse transcription polymerase chain reaction analysis confirmed that the transfection of wtMEK1 into NHK cells increased cystatin A mRNA levels, whereas the transfection of dn-MEKK1, dn-MEK1, or dn-JNK decreased them (Fig. 7). The transfection of dn-MKK3, dn-MKK4, or dn-MKK7, or dn-p38 was without effect (data not shown). These results indicate that (i) the MEK1/MKK7/JNK pathway induces up-regulation of cystatin A promoter activity, (ii) MEK1 is functionally located upstream of MKK7 and JNK in this cascade, and (iii) activa-
tion of the MEKK1/MKK7/JNK pathway is distinct from the inhibitory pathway that depends on Raf-1/MEK1/ERK.

The MAPK-responsive Element Is Identical to the TPA-responsive Element, T2, of the Cystatin A Gene—Previous analyses revealed that the cystatin A promoter contains at least two putative TPA-responsive elements, T1 (−189 to −196) and T2 (−272 to −278), and T2 is most likely critical for TPA-dependent stimulation of cystatin A promoter activity (14). To determine the critical region(s) for MAPK-dependent positive and negative regulation of cystatin A expression, T1- and/or T2-deleted constructs were co-transfected into NHK cells together with various MAPK vectors (Fig. 8). Deletion of T1 had no effect on the promoter activity; the T1-deleted vector allowed the retention of dn-MEK1, dn-ERK1, and wtMEKK1-dependent stimulatory effects on cystatin A promoter activity. In contrast, the T2-deleted vector resulted in a loss of stimulatory effects of dn-MEK1, dn-ERK1, and wtMEKK1 (Fig. 8). These results indicate that the T2 region of the cystatin A gene is critical for the regulation of not only the stimulatory MEKK1/MKK7/JNK pathway but also the inhibitory Raf-1/MEK1/ERK pathway.

Use of Adenovirus Vectors Confirms the Stimulatory Effects of da-Ras and da-ERK on Cystatin A Expression—Because the Lipofectin method is not efficient enough for detecting
changes in cystatin A mRNA levels by Northern blot or protein levels by Western blot in NHK cells, an adenovirus vector-dependent transfection system was utilized. Transfection of adenovirus vector containing da-ras (AxCAAdrAs) increased cystatin A mRNA and protein levels (Fig. 9, lane 3). The transfection of adenovirus vector containing a dominant negative form of ERK1 (AxCAAdnERK1) also increased cystatin A mRNA and protein levels (Fig. 9, lane 4). The co-transfection of AxCAAdrAs and AxCAAdn-ERK1 resulted in a more marked increase in cystatin A promoter activity (Fig. 9, lane 5). In contrast, the transfection of dominant negative MEKK1-expressing adenovirus vector (Ad-dn-MEKK1) suppressed the cystatin A expression. These results indicate that the activation of Ras does indeed stimulate cystatin A expression in NHK cells, which can be suppressed by the Raf-1/MEK1/ERK pathway.

Differential Distribution of Active ERK and JNK in Normal Human Epidermis—Immunohistochemical analyses were performed to locate active ERK and JNK proteins in the normal human epidermis. ERKs were expressed in nuclei of basal cell layers and cytoplasm of suprabasal cell layer (Fig. 10A). The active phosphorylated forms of ERKs were present exclusively in the nuclei of the basal cell layer (Fig. 10B). This suggests that cystatin A expression is suppressed because of the activated Raf-1/MEK1/ERK pathway in the basal cell layer. On the other hand, JNK was detected in both cytosole and nuclei of upper spinous cell and granular cell layers (Fig. 10C). Furthermore, active phosphorylated JNK was found in the nuclei of upper spinous cell and granular cell layers (Fig. 10D). This suggests that cystatin A expression is stimulated because of the activation of MEKK1/MMK7/JNK pathway as well as loss of inhibitory tonus via the Raf-1/MEK1/ERK pathway. All these results are consistent with the suprabasal expression pattern of cystatin A in the normal human epidermis (47).

AP-1-dependent Regulation of Cystatin A Expression in Human Epidermis—Our results clearly demonstrated that AP-1-dependent cystatin A expression in the human epidermis is regulated by two MAP kinase systems: positively via MEKK1/MMK7/JNK and negatively via Raf-1/MEK1/ERK. Although both pathways are triggered by the activation of Ras, the differential localization of downstream targets (active ERK in the basal epidermis and active JNK in the upper epidermis) explains the suprabasal expression of cystatin A in the normal human epidermis.

AP-1 is comprised of Jun and Fos protein families. Previously we reported that c-Fos and c-Jun and/or Jun D are involved in cystatin A expression (44). Because active JNKs efficiently phosphorylate c-Jun and less efficiently phosphorylate Jun D, but do not phosphorylate Jun B at all, it is conceivable that c-Jun and/or Jun D are the regulatory factors for cystatin A expression (44). It should be noted that c-Fos is constitutively expressed, whereas c-Jun is marginally expressed in the normal human epidermis (45). This suggests that c-Jun is more critical than c-Fos for AP-1-dependent regulation in normal human epidermis. It is known that the phosphorylation of c-Jun by JNK reduces its susceptibility to pro teaseasme attack, increasing its half-life (46).

The activation of the Raf-1/MEK1/ERK pathway is usually associated with cell proliferation (19). On the other hand, terminal differentiation of keratinocytes is associated with growth arrest associated with the activation of other MAP kinases (50). In addition to TPA, calcium also induces keratinocyte differentiation, and our preliminary analysis suggested that high calcium concentrations (1.0 mM) decreased phosphorylated ERKs in NHK cells (data not shown). This is consistent with the finding that cystatin A expression is increased by calcium treatment in NHK cells (6).

TPA mediates its effects through the activation of PKC. Previously we reported that the expression of cystatin A is stimulated by PKC-α (14). Interestingly, the expression of involucrin, another CE precursor protein, is also stimulated by PKC-α and PKC-γ (51) as well as by calcium. Recently Ng et al. (52) reported that the calcium-responsive region of the involucrin gene is regulated by AP-1. Although these effects suggest that the expression of cystatin A and involucrin is regulated by a similar mechanism, Efimova et al. (25) reported that involucrin gene expression in keratinocytes is stimulated by the PKC/Ras/MEKK1/p38 pathway but not by the JNK pathway. This implicates distinctive MAP kinase-dependent regulatory mechanisms among the keratinocyte differentiation markers.

In the present study, we have characterized the nature of cystatin A expression in keratinocytes. The results demonstrate that (i) the stimulatory effect of TPA on cystatin A expression is mediated through Ras, (ii) the Ras/MEKK1/MMK7/JNK/AP1 and Raf-1/MEK1/ERK pathways mediate stimulation and inhibition of cystatin A promoter activity, respectively, and (iii) these pathways depend on an intact T2 region (−272 to −278) of the cystatin A gene. Our results define another novel TPA-dependent MAP kinase-mediated regulatory mechanism for keratinocyte differentiation.

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