Phorbol Ester Stimulated Cathepsin L Expression in U937 Cells

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Abstract
Cathepsin L (ctsl) is a lysosomal cysteine proteinase, the synthesis and secretion of which is induced by transformation, growth factors, and tumor promoters. We studied the effect and the mechanism of action of phorbol ester (TPA) on the expression of ctsl mRNA in U937 histiocytic leukemia cells. TPA treatment induces ctsl mRNA in a manner that is dose-dependent, occurs at the level of transcription, and is ablated by cotreatment with cycloheximide but is unaffected by dexamethasone. Treatment with TPA plus staurosporine, a potent protein kinase C inhibitor, results in greater expression of ctsl mRNA than does treatment with TPA alone. Similar to TPA, staurosporine alone increases ctsl transcription, an effect that is inhibited by cycloheximide. Another PKC inhibitor, H7, exerted no effect upon the induction of ctsl mRNA by either TPA or staurosporine. Staurosporine and H7, however, inhibit the increase in c-jun mRNA by TPA. In contrast, the tyrosine kinase inhibitors herbimycin A and genistein inhibit the effect of TPA and staurosporine upon ctsl mRNA with little or no effect on c-jun expression. Pretreatment with sodium orthovanadate enhances the induction of ctsl expression by TPA and staurosporine. These data suggest that, in U937 cells, TPA-stimulated ctsl gene transcription is apparently activated by a protein kinase C-independent signal transduction pathway involving tyrosine kinase activation.

Introduction
The lysosomal protease ctsl is widely distributed in many cells and tissues and is produced at high levels in many human tumors (1–6). Cathepsin L is an acidic cysteine protease that plays a major role in intracellular catabolism and also cleaves extracellular proteins such as fibronectin, collagen, elastin, and laminin (7–9). Activated murine and human macrophages synthesize large amounts of cathepsin L (10, 11). Peripheral blood monocytes that differentiate to macrophages express high levels of ctsl, which is partly responsible for their elastinolytic activity (4). Cathepsin L can interfere with antigen presentation by macrophages in vitro (10), and ctsl expression is elevated in the infiltrating macrophage of antigen-induced arthritis (4). In murine NIH3T3 cells, ctsl mRNA levels are increased by TPA treatment, and this increase is regulated at the level of transcription (12–14). In addition, this stimulation is inhibited by cycloheximide, thus placing ctsl in a class of TPA-regulated genes that require protein synthesis to induce transcription (13).

Treatment of U937 cells with TPA activates PKC, resulting in the stimulation of c-jun and c-fos proto-oncogene expression (15, 16). The increased expression of these genes leads to activation of other genes that contain the AP-1 element, resulting in the induction of monocyctic differentiation (17). The stimulation of c-jun/c-fos by TPA in U937 cells is inhibitable by ST and other PKC inhibitors (16). The increased expression of c-jun/c-fos does not require protein synthesis and is inhibited by the glucocorticoid dexamethasone (15). Our preliminary results showed that TPA transcriptionally increases ctsl mRNA in U937 cells and that this increase is inhibited by cycloheximide but not by dexamethasone. Consequently, we hypothesized that the effect of TPA on ctsl expression in U937 cells may be mediated by PKC but that the pathway diverges subsequently, becoming different from that controlling c-jun/c-fos expression. Our studies imply that the induction of ctsl mRNA by TPA does not require PKC activation since inhibitors of PKC fail to reduce the response to TPA. Indeed, ST acts to stimulate ctsl mRNA expression. Inhibitors of TK and tyrosine phosphatase repress and enhance, respectively, the stimulation of ctsl mRNA by TPA.

Results
Low concentrations of TPA stimulate expression of ctsl mRNA in U937 cells (Fig. 1). The steady-state level of ctsl mRNA exhibited a dose-dependent increase from 2-fold at 0.1 nM to greater than 15-fold at 5 nM. Kinetic analysis of the effect of 2.5 nM TPA treatment of U937 cells indicates that ctsl mRNA expression is induced within 3 h of treatment, reaching 5-fold by 6 h and 14-fold by 12 h (Fig. 2). ctsl mRNA levels remain elevated for up to 72 h (data not shown). The protein synthesis inhibitor, Chx (10 μg/ml), completely inhibits the TPA-mediated increase in ctsl steady-state mRNA expression (Fig. 3). In contrast, dexamethasone (1 μM) treatment of U937 cells exerts no effect upon ctsl mRNA expression, either alone or in combination with TPA (data not shown). Nuclear run-off assays show that TPA induces ctsl gene transcription and that this effect is inhibited by Chx (Fig. 4).

Because many TPA effects are ascribable to protein kinase C activation, we sought to determine whether the TPA

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3 The abbreviations used are: ctsl, cathepsin L; TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; Chx, cycloheximide; ST, staurosporine; OAG, 1-oleoyl-2-acetyl-glycerol; Van, sodium orthovanadate; IL-1, interleukin; gapdh, glyceraldehyde phosphate dehydrogenase; TK, tyrosine kinase.

4 S. Weiss, personal communication.
mediated stimulation of ctsl mRNA is PKC dependent. TPA treatment (2.5 nM) of U937 cells increased ctsl mRNA about 5-fold (Fig. 5A). Cells treated simultaneously with TPA and the protein kinase inhibitor ST (10 nM; Ref. 18) exhibited approximately a 9-fold increase in ctsl mRNA (Fig. 5A). The level of ctsl expression in cells treated with ST alone was approximately 5-fold greater than control, indicating that ST has agonist effects and that cotreatment results in an additive effect on ctsl mRNA expression. The induction of ctsl mRNA by ST is dose dependent, with increases in ctsl mRNA occurring at concentrations as low as 5 nM and as high as 100 nM (Fig. 6). Similar to cotreatment with TPA, cycloheximide inhibited the effect of ST upon ctsl mRNA (Fig. 3), whereas dexamethasone did not alter the response (data not shown). Staurosporine also enhanced transcription of the ctsl gene, which was also inhibited by Chx (Fig. 4).

Cell growth was not significantly affected during ST treatment.

The protein kinase inhibitor H7 (10 μM; Ref. 19) exerted no independent effect and did not inhibit either the TPA-mediated increase in ctsl mRNA or that seen with ST.
Fig. 6. Dose-response of ctsl mRNA expression to ST. U937 cells (5 × 10⁵/ml) were treated with concentrations of ST as indicated for 6 h. Total cellular RNA (20 μg/lane) was analyzed by Northern hybridization to 32P-labeled human ctsl and chicken gapdh DNA probes.

Fig. 7. Effect of protein kinase inhibitors on the expression of c-jun in response to TPA. U937 cells (5 × 10⁵/ml) were pretreated for 15 min with or without 10 nM ST or 10 μM H7 (A) or 2 μM herbimycin A or 200 μM genistein (B), followed by the addition of 20 nM TPA for 1 h. Total cellular RNA (20 μg/lane) was analyzed by Northern hybridization to 32P-labeled human c-jun cDNA probe.

treatment (Fig. 5B). The more specific PKC inhibitors, chelerythrine (10 μM; Ref. 20) and GF109203X (50 μM; Ref. 21), similarly had no independent effect and did not ablate the induction of ctsl mRNA by either ST or TPA (data not shown). The TPA-mediated increase in the expression of c-jun mRNA is PKC dependent and can be inhibited by ST and H7. Treatment of cells with 20 nM TPA for 1 h induced c-jun mRNA expression greater than 7-fold (Fig. 7A), and this increase was inhibited by ST (10 μM) and H7 (10 μM). Because the kinetics of the induction by TPA are different for c-jun and ctsl, it is possible that the effectiveness of ST is reduced with time. To address this, cells were treated with ST for 5 h, followed by an additional 1-h treatment with TPA. The expression of c-jun was inhibited to an extent similar to that seen with a simultaneous TPA/ST treatment for 1 h (data not shown). Treatment of U937 cells with the diacylglycerol analogue OAG (250 μM; Ref. 22) and the calcium ionophore A23187 (2 μM; Ref. 23), either alone, in combination, or with TPA, did not alter the expression of ctsl mRNA (data not shown). In addition, pretreatment of the cells for 20 h with OAG did not prevent the subsequent induction of ctsl mRNA by TPA but did inhibit the subsequent induction of c-jun by TPA (data not shown).

Because there was no clear result suggesting an essential role of PKC or another serine/threonine kinase in the TPA-mediated response, we tested the effect of TK inhibitors on ctsl mRNA expression. Herbimycin A (2 μM; Ref. 24) and genistein (200 μM; Ref. 25), while without independent effect on basal ctsl mRNA (data not shown), markedly inhibited both the ST and TPA-mediated increases in ctsl mRNA expression in U937 cells (Fig. 5C). Herbimycin marginally enhanced, and genistein slightly inhibited, the induction of c-jun mRNA by TPA (Fig. 7B). Treatment of U937 cells with the tyrosine phosphatase inhibitor Van (50–500 μM) did not alter the expression of ctsl mRNA (data not shown). When the cells were pretreated with Van for 30 min, followed by TPA (2.5 nM), there was a dose-dependent increase in the expression of ctsl over that seen with TPA alone. At 200 μM Van, the increase was greater than 75%, above that with TPA alone (Fig. 8). The combination of ST and Van showed a similar dose-dependent increase in ctsl expression above ST treatment (data not shown).

Since phorbol ester can alter tyrosine phosphorylation in U937 cells within minutes (26), we examined the temporal relationship between the induction of ctsl mRNA by TPA and the inhibition by herbimycin and cycloheximide (Fig. 9). Addition of herbimycin up to 3 h after initiation of TPA treatment inhibited the stimulation of ctsl mRNA. Loss of inhibition occurred if cycloheximide was added 2–3 h after the TPA.

Discussion

In this study, we have characterized the action of TPA upon ctsl mRNA expression in the human leukemic U937 cell line. The stimulation of ctsl mRNA expression by TPA is consistent with that seen in murine fibroblasts; it occurs at the level of transcription and requires protein synthesis (12, 14). In U937 cells, the increase in ctsl gene expression is quite sensitive to TPA, requiring as little as 0.1 nM for an effect. This induction is dose dependent and is maximal between 5 and 20 nM. The kinetics of this response are
relatively slow, contrasted with c-jun and c-fos mRNA expression. Elevation of ctsl mRNA requires 3 h, whereas increases in c-jun and c-fos mRNA levels occur in as little as 15 min (16). The ctsl mRNA level peaks between 6 and 12 h and persists for as long as 72 h of TPA treatment. In contrast, c-jun/c-fos mRNA levels return to baseline after 1–2 h (16, 27). Chx inhibits the stimulation of ctsl mRNA expression by TPA in U937 cells, an effect comparable to the inhibition by Chx of the TPA-induced increase in transcription of the insulin-like growth factor gene in U937 cells (28). ctsl thus belongs to a class of genes whose regulation of expression by TPA requires de novo protein synthesis.

The effects of TPA treatment in many cells, including U937 cells, are reported to be mediated by PKC activation, followed by induction of expression of the proto-oncogenes c-jun and c-fos (16, 17, 27, 29–31). Subsequently, there is activation of a number of AP-1 responsive genes (27). In U937 cells, as in other cells, the induction of c-jun/c-fos occurs independently of protein synthesis (27). Dexamethasone inhibits induction of the proto-oncogenes and many of the subsequent cellular events associated with TPA activation, including transcription mediated via the AP-1 element (15, 31). We have shown, on the other hand, that TPA induction of ctsl mRNA expression requires protein synthesis and is not inhibited by dexamethasone. Therefore, the induction of ctsl gene transcription in U937 cells possibly occurs independently of the AP-1 element.

Since dexamethasone does not inhibit PKC activation, this raised the possibility that the TPA-stimulated pathway leading to the induction of c-jun/c-fos and ctsl diverges downstream of PKC. Treatment of the cells with PKC inhibitors suggests that the pathways may be distinct: one dependent, the other independent of PKC. This conclusion is strongly supported by the fact that ST, H7, and two other specific PKC inhibitors do not inhibit the induction of c-jun mRNA expression by TPA, whereas these agents do inhibit the induction of c-jun mRNA expression by TPA. It is possible that TPA is acting through an isoform of PKC that is not sensitive to ST (or the other inhibitors). However, of the four isoforms reported to be expressed in U937 cells (32), ST is able to inhibit the α, β, and ε isoforms (33), and the ζ isoform does not bind diacylglycerol or phorbol ester (34). Furthermore, inhibition of the induction of c-jun, but not ctsl, by TPA when U937 cells are pretreated with OAG provides evidence that the pathways of activation of c-jun and ctsl gene expression by TPA are distinct. Therefore, under conditions that clearly inhibit the PKC-dependent induction of c-jun expression by TPA, we have been able to show that TPA is able to induce ctsl. Further research investigating the expression of individual PKC isoforms and their sensitivities to the various agonists and inhibitors will be necessary to definitively assess PKC involvement.

ST not only fails to inhibit the stimulation of ctsl expression by TPA, it also acts as an agonist, and there is an additive effect upon ctsl mRNA expression of combined ST/TPA treatment. Similar to the stimulation of ctsl mRNA expression by TPA, the activation by ST is dose dependent, inhibited by Chx, occurs via increased transcription, and is not altered by dexamethasone. Although ST is widely used as a PKC inhibitor, it exerts agonist effects upon prostaglandin E2 production (35), expression of IL-2, IL-4, and IL-8 (36, 37), and cell proliferation (38). Our results demonstrate that, in U937 cells, ST can exert agonist effects at the same time it is inhibiting other putative PKC-dependent responses and that there are TPA-mediated effects that are not inhibitable by ST.

The TK inhibitors, herbimycin A (24) and genistein (25), significantly inhibit the induction of ctsl mRNA expression by both ST and TPA. Although genistein was able to inhibit partially the increased expression of c-jun by TPA, herbimycin consistently, albeit slightly, enhanced it. These results further imply that a TK(s) different than that associated with c-jun activation mediates the effect of TPA on ctsl. The enhancement of ctsl expression with vanadate is consistent with the activation of a TK. Treatment of hematopoietic cells, including U937 cells, with a tyrosine phosphatase inhibitor augments the constitutive level of tyrosyl phosphorylation (39). Although we saw no effect of the vanadate alone, others have demonstrated that pretreatment of cells with this inhibitor was associated with increased ability to detect tyrosyl phosphorylation of phospholipase C-γ (40) and the TPA-stimulated TK-dependent activation of Ras-GTP (41).

The activation of TK by TPA and ST has been demonstrated previously. Herbimycin A inhibits the disruption of actin filaments by TPA in FL and MDCK cells (42) and suppresses gap junctional intercellular communication in 3T3-L1 cells (43). The stimulation of BALB/c proliferation by TPA, which can be augmented by ST, is inhibited by the TK inhibitor tyrphostin AG490 (38). In PC12 cells, genistein inhibits the PKC-dependent activation of Ras-GTP by TPA (41), and TPA induces the tyrosyl phosphorylation MAP kinase in both PC12 (44) and U937 (26) cells. However, it is unlikely that induction of ctsl gene expression by TPA is mediated by activation of MAP kinase, since such activation occurs in minutes independently of protein synthesis, is calcium sensitive, and requires PKC (26, 45–48). None of these conditions pertains to the stimulation of ctsl in U937 cells.

Addition of herbimycin, but not Chx, 3 h after initiation of TPA treatment inhibited the stimulation of ctsl mRNA, and vanadate by itself exerted no effect on ctsl expression. Therefore, protein synthesis appears to precede the acti-
viation of TK that leads to the induction of ctsl mRNA. Preliminary Western blot analysis (data not shown) implicates the phosphorylation of several proteins in a manner corresponding to the Northern analysis, i.e., stimulation by both TPA and ST and inhibition of phosphorylation by Chx, herbimycin, and genistein cotreatment. This suggests that TPA may be inducing the production of a TK or a factor that activates the TK. Additional studies will be necessary to characterize the TKs and targets of phosphorylation involved in the induction of ctsl.

In summary, TPA and ST stimulate ctsl mRNA expression in U937 cells via a transcriptional mechanism that requires protein synthesis and is not inhibited by dexamethasone. This regulation of expression requires tyrosine kinase activity and appears to occur in the absence of PKC activation. These observations indicate the presence of an alternative, TK-mediated, phorbol-ester-stimulated signal transduction cascade and reinforce the complexity of intracellular signaling processes.

Materials and Methods

Reagents. The phorbol ester TPA, ST, OAG, A23187 (Sigma Chemical Co., St. Louis, MO), herbimycin A, genistein (National Cancer Institute, Drug Synthesis and Chemistry Board, Bethesda, MD), lavendustin A, tyrophostin (GIBCO-BRL, Gaithersburg, MD), H7, and the bisindolylmaleimide GF 109203X (Calbiochem, San Diego, CA) were dissolved in DMSO. Chx and Van (Sigma) were dissolved in water. Dexamethasone (Sigma) was dissolved in ethanol. Mouse monoclonal anti-phosphotyrosine antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

Cells. U937 cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 supplemented with 10% FCS, 5 mm l-glutamine, penicillin (50 units/ml), and streptomycin (50 µg/ml) at 37°C and 5% CO2. The cells were passaged once every 2–3 days. Cells were seeded at 5 x 105/ml in 100-mm plates at the beginning of each experiment.

RNA Isolation, Northern Blots, and Nuclear Run-Off Assay. U937 cells at the end of each experimental period were centrifuged at 1000 x g for 5 min. Total cellular RNA was extracted using a modified guanidinium thiocyanate procedure (49). Total RNA (20 µg) was denatured, fractionated on a 2% formaldehyde-agarose gel, and transferred onto a nitrocellulose membrane (Schleicher & Schuell). The blots were then hybridized with radiolabeled probes generated from randomly primed cDNA for human ctsl (1.2-kb Drai/DraI fragment of the ctsl cDNA), human proto-oncogene c-jun (2.0-kb EcoRI fragment of pGEM7zj), and chicken gapdh (1.2-kb PstI fragment of pGAD-28). Northern blots were washed according to the manufacturer (Schleicher & Schuell). For nuclear transcription assays, nuclei were isolated from cells, and mRNA was labeled with 32P-UTP. The insert cDNAs (see above) for ctsl and gapdh were separated from the plasmid DNA on an agarose gel and Southern blotted to nitrocellulose strips. The labeled RNA was hybridized as described (50) to filter strips containing only the cDNA probe. After hybridization, nuclear blots were washed and treated with RNase A as described (51). All blots were exposed to Kodak X-OMAT film with intensifying screens at −80°C and quantitated with a Betascanner (Betagen). All of the results of ctsl and c-jun expression are normalized based upon gapdh expression (gapdh expression shown only for Figs. 1 and 6). All treatment conditions were examined a minimum of three times.

References


