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Cathepsin S (CTSS) is a cysteine protease that is constitutively expressed in APCs and mediates processing of MHC class II-associated invariant chain. CTSS and the Ets family transcription factor PU.1 are highly expressed in cells of both myeloid (macrophages and dendritic cells) and lymphoid (B lymphocytes) lineages. Therefore, we hypothesized that PU.1 participates in the transcriptional regulation of CTSS in these cells. In A549 cells (a human epithelial cell line that does not express either CTSS or PU.1), the expression of PU.1 enhances CTSS promoter activity ~5- to 10-fold. In RAW cells (a murine macrophage-like cell line that constitutively expresses both CTSS and PU.1), the expression of a dominant-negative PU.1 protein and a short-interfering RNA construct attenuates basal CTSS promoter activity, mRNA levels, and protein expression. EMSAs show binding of PU.1 to oligonucleotides derived from the CTSS promoter at two different Ets consensus binding elements. Mutation of these sites decreases the baseline CTSS activity in RAW cells that constitutively express PU.1. Chromatin immunoprecipitation experiments show binding of PU.1 with the CTSS promoter in this same region. Finally, the expression of PU.1, in concert with several members of the IFN regulatory factor family, enhances CTSS promoter activity beyond that achieved by PU.1 alone. These data indicate that PU.1 participates in the regulation of CTSS transcription in APCs. Thus, manipulation of PU.1 expression may directly alter the endosomal proteolytic environment in these cells. The Journal of Immunology, 2006, 176: 275–283.

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enhancer. Igα enhancer, CD20, IL-1β, IL-18, and scavenger receptor, contain composite PU.1/IRF-binding elements in which a PU.1 site is located just 5′ to the IRF consensus element. Specifically, PU.1/IRF-4 complexes bind to DNA via the DNA binding domain of both proteins and associate with others via the proline-, glutamine-, serine-, and threonine-rich domain of PU.1. After phosphorylation of PU.1 at serine 148, this complex binds DNA and promotes transcriptional activity (20). Both IRF-4 and IRF-8 can synergize with PU.1 in the human IL-1β promoter to increase gene activity (21). IL-1β gene activity dramatically increases with the supplementation of IRF-1 to transfected 3T3 fibroblasts in addition to either PU.1-IRF-4 or PU.1-IRF-8. Thus, PU.1 can complex with several members of the IRF family to promote gene expression via several different structural mechanisms.

The constitutive expression of CTSS occurs in several cell types that also exhibit high expression of PU.1, including B cells, dendritic cells, and macrophages. In addition, analysis of the CTSS promoter region reveals the existence of several consensus binding sites for PU.1, with at least two of these sites located near the CTSS promoter ISRE (6). Based on these observations, we postulated that PU.1 may participate in the transcriptional regulation of CTSS expression in professional APCs, and that this may involve protein-protein interactions with IRF family members. We now show that PU.1 can physically bind to and enhance CTSS promoter activity in epithelial cells. Furthermore, IRF family members augment PU.1-dependent activation of this promoter. Manipulation of PU.1 activity may directly alter the lysosomal proteolytic environment and therefore modulate the proteolytic processes mediated by CTSS.

Materials and Methods

Cells and materials

The type II alveolar epithelial (A549) cell line (American Type Culture Collection; ATCC), the 293T epithelial cell line, the HL-60 human promyelocytic leukemia cell line (ATCC), and the mouse RAW264.7 (RAW; ATCC) cell line were cultured in DMEM supplemented with 10% heat-inactivated FBS (Invitrogen Life Technologies), 10 mM glutamine, and penicillin/streptomycin.

Plasmids and transient transfection analysis

An 892-bp fragment of the CTSS gene (CTSS −859/+32) was cloned into PGL2 basic (Promega) as previously described (6). CTSS (−859/+32) was used as a template to generate truncated CTSS 5′-flanking regions by PCR, designated CTSS (−422/+32), CTSS (−230/+32), and CTSS (−75/+32). The full-length construct, CTSS (−859/+32), was also used as a template to mutate the PU.1 consensus sites at −48, −139, −587/−593, −663, −771, and −795 (GGAA/T to GAGA/T; QuikChange XL Site-Directed Mutagenesis Kit; Stratagene). The PU.1 expression vector (pcDNA1-PU.1) and the dominant negative (DN)-PU.1 expression vector (pcDNA-PN-PU.1) were gifts from Dr. P. Auron (University of Pittsburgh School of Medicine, Pittsburgh, PA) and have been previously described (22, 23). The pcDNA-PN-PU.1 plasmid was generously provided by Dr. N. Sibinga (Albert Einstein College of Medicine, Bronx, NY) and has been previously described (22, 23). The pcDNA-IRF-1 plasmid was generously provided by Dr. N. Sibinga (Albert Einstein College of Medicine, Bronx, NY) and has been previously described (22, 23). The IRF-1 and IRF-2 expression vectors (pcDNA-IRF-1 and pcDNA-IRF-2) have been previously described (22, 23). The IRF-4 expression construct (pcDNA3.1-IRF-4) was a gift from Dr. L. Gilmcher (Harvard School of Public Health and Medicine, Boston, MA), and the IRF-8 expression construct (pcDNA3.1-IRF-8) was a gift from Dr. H. Singh (Howard Hughes Medical Institute, Chicago, IL). The IRF-3, IRF-5, and IRF-6 cDNAs were obtained from the ATCC and subcloned into pcDNA3.1 using standard techniques. The murine wild-type NO synthase (NOS)2 promoter/reporter construct (inducible NOS (−1485/+31)) in PGL2 basic has been previously described (25, 26). DNA sequencing was undertaken to confirm the accuracy of all constructs used in this study.

Transient transfections were conducted in A549, RAW, and 293T cells that were grown to 70% confluence in 60-mm dishes with 6 µl of FuGene-6 (Roche) and 2 µg of total DNA. Total plasmid DNA was kept constant for all experiments by the addition of empty vectors. Transfected cells were incubated for 48 h, placed in lysis buffer (Promega), and subjected to one freeze-thaw cycle. Firefly luciferase activity was then assayed in lysates using the luciferase assay method (Promega). In all experiments, transfection efficiency was monitored by cotransfection with 0.25 µg of pSV-β-galactosidase (Promega) according to the manufacturer’s instructions. PU.1 cotransfection studies were not normalized, because overexpression of PU.1 enhanced pSV-β-galactosidase expression. Transfection studies were performed in an identical fashion with the murine NOS2 promoter and were normalized for β-galactosidase activity.

Generation of short-interfering RNA (siRNA) PU.1 RAW cell lines

To construct PU.1 RNAi plasmids, we obtained pSUPER.retro.puro plasmid from OligoEngine and followed the manual provided by the manufacturer. Briefly, two 19-nt target sequences for siRNA were empirically chosen. Two candidate sequences were located −115 bp from the start and 150 bp from the end of open reading frame of murine PU.1, both of which are started with AA at their 5′ ends. To ensure no significant sequence homology with other genes, the sequences were analyzed by BLAST search. Based on the criteria described above, two sets of complementary oligonucleotides were designed to generate hairpin dsRNAs. The first set of 64-nt long sequences that encompass +115 to +135 are as follows: forward, 5′-GATCCCCCATATCGACATCTGTTCCAGACAGACAGTAAGATCTGCTAGGGG-3′; reverse, 5′-AGCTTTCACAAAGGACCTAGGCTACTCCTGTCTCAGGATGTAATGGTGCATGTCATGAGGGG-3′. The second set of 64-nt long sequences that encompass +650 to +670 are as follows: forward, 5′-GATCCCGGAGATGACCTACCTACAGCAGTCAGTCGATG TTCGTCTTACAA-3′; reverse, 5′-AGCTTTTTGAATTCACCCGACATCTGTTCCAGACAGACAGTAAGATCTGCTAGGGG-3′. Underlines indicate PU.1-specific sequences. To generate stable cell lines that express PU.1 siRNA, RAW 264.7 cells (0.5 × 10⁶ cells) were transfected with PU.1 RNAi plasmids by GenePORTER 2 (Gene Therapy Systems) as specified by the manufacturer. Transfected cells were grown for 2 days before treatment with 2 µg/ml puromycin (Sigma-Aldrich). Candidate colonies were screened by PU.1 immunoblotting.

Immunoblot analysis and active site labeling of CTSS

RAW cell line were grown to 80% confluence in 10-cm culture dishes. Cells were lysed, and extracts were run on 12% SDS-PAGE. Fifty micrograms was loaded in each lane for PU.1, and 25 µg was loaded in each lane for CTSS. Proteins were transferred to polyvinylidene difluoride membranes (Millipore) and probed with specific Abs. The CTSS Ab was a gift from Dr. G. Shi (Brigham and Women’s Hospital, Boston, MA) and has been previously described (12), and the PU.1 Ab was obtained from Santa Cruz Biotechnology (Sp1-7, T-21). Membranes were incubated with the appropriate secondary HRP-conjugated antisera (Pierce) and detected by chemiluminescence (Pierce). To ensure equal loading of protein, the PU.1 gel was stripped and reprobed with an Ab against actin (Santa Cruz Biotechnology; I-19) or GAPDH (Abcam).

For the active labeling of CTSS, RAW cells were lysed in 50 µl of 1% Triton X-100, 50 mM sodium acetate (pH 4.2), 1 mM EDTA, and 3 mM DTT on ice for 30 min. Post-nuclear extracts (NE) were normalized to total protein content and incubated with JPM565-biotin (gift from H. Ploegh, Massachusetts Institute of Technology, Boston, MA) for 1 h at 37°C (13). Labeled lysates were then subjected to 12% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and visualized by incubation with streptavidin-HRP (Pierce), followed by chemiluminescence.

Northern blot

RAW cell lines were grown to 80% confluence in 10-cm culture dishes, and total RNA was extracted using the RNeasy Mini kit (Qiagen). Eight micrograms of total RNA was fractionated on a formaldehyde-agarose gel, transferred to a nylon membrane, and probed with a 32P-labeled CTSS probe as previously described (6). The cDNA probe was labeled with [α-32P]dCTP using the Prime-It II Random Primer labeling kit (Stratagene). The membrane was hybridized using QuickHyb solution (Stratagene) at 68°C for 60 min, washed according to the manufacturer’s directions, and exposed to Kodak XAR film at −80°C. Equity of sample loading was assessed by stripping and reprobing the membrane with 32P-labeled 28S probe.

EMSA

NE from RAW cells were prepared using a modification of the method described by Dignam et al. (6). Double-stranded oligonucleotides derived from the PU.1 consensus binding sites (−48, 5′-ATGGGACA AGTTCCAAATTTCTT; −139, 5′-AAGACCATAGGGGAAATGACTA...
GAGGT; −221, 5′-AAAGACGGT GGATATAGGCT; −524, 5′-AACATGATGGTTTACGTTAAGGTT; −567, 5′-AAAGACGTTG GTTTGGGGAA GTCT; −587/593, 5′-AAAGACGTTTTCCTAG GAAATCATCA A; −663, 5′-GATGAA GAAAGAAGGCT GAGTGGAAC; −771, 5′-GTGATCTTTTTT CCTGATATAAC; −795, 5′-AACATCTTACATGGATATATGGGT) were radiolabeled by filling in a 5′ overhang with [α-32P]dCTP or -dTTP and Klenow fragment (New England Biolabs) and purified by gel filtration (Chromatography Spin columns; Amersham Biosciences). ChIP analysis was performed as described previously (28) using the Chromatin Immunoprecipitation assay (ChIP).

Pu.1 protein was generated from the pcDNA3-Pu.1 plasmid described above, with empty pcDNA3 plasmid as a control, using the TNT (in vitro transcription/translation products) T7 Quick Coupled Transcription/Translation system (Promega) and was confirmed by SDS-PAGE. Double-stranded nucleotides derived from the consensus Pu.1 consensus binding site were described and contained corresponding mutants, with mutant base pairs underlined. (−48 mut, 5′-ATGTGACAGTGTCATTAATTTTC; −139 mut, 5′-AAGCACTAGGAATGACATGG) were annealed, end-labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (NEN Life Science Products), and purified by gel filtration (GS-50 spin columns; Amersham Biosciences). EMSA were performed as previously described (25, 27) with protein-DNA binding reactions containing 20,000 cpm of DNA probe for 30 min at room temperature. For cold competition assays, unlabeled double-stranded identical and nonidentical (consensus AP-1 binding site) competitors were added at a 200-fold molar excess to the radiolabeled probe. Supershift Abs (200-fold molar excess to the radiolabeled probe) were added before the addition of radioactive probe, as described above.

Chromatin immunoprecipitation assay (ChIP)

ChIP analysis was performed as described previously (28) using the Chromatin Immunoprecipitation Assay Kit (Upstate Cell Signaling Solutions) on 293T Pu.1 transfectants and HL-60 cells, a human promyelocytic cell line that constitutively expresses Pu.1 (29). 293T (2 × 10⁵) cells were plated into each well of a 6-well plate and incubated overnight. Cells were transfected with the Pu.1 expression vector or the empty vector as a control and grown for another 48 h. Proteins were cross-linked to the genomic DNA by addition of formaldehyde (1%, v/v) for 10 min at 37°C. The cells were washed twice, pelleted by centrifugation, resuspended in lysis buffer (50 mM Tris-HCl (pH 8.1), 10 mM EDTA, and 1% SDS) supplemented with protease inhibitors, and incubated for 10 min on ice. Cell lysates were sonicated three times for 30 s each time to shear the genomic DNA to lengths between 200 and 1000 bp. Supernatants were collected and diluted 1/10 with dilution buffer (167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, and 16.7 mM Tris-HCl (pH 8.1)) supplemented with protease inhibitors. Samples were precleared with 75 μl of salmon sperm DNA/protein A agarose-50% slurry for 30 min at 4°C. Supernatants were then incubated overnight at 4°C either with 10 μg of specific Abs against Pu.1, GATA1, and ETS1 (Santa Cruz Biotechnology).

Results

Pu.1 regulates CTSS promoter activity

CTSS and Pu.1 are highly expressed in professional APCs, including B cells, dendritic cells, and macrophages. To determine whether Pu.1 can up-regulate CTSS promoter activity in cells that do not constitutively express either CTSS or Pu.1, A549 cells were cotransfected with the full-length CTSS promoter-reporter construct (CTSS (−859/+32)) and an expression vector containing Pu.1 cDNA. Control cells were transfected with the empty expression vector to normalize for total DNA in each experiment. The expression of Pu.1 in A549 cells results in a 5- to 10-fold increase in CTSS promoter-reporter activity as assessed by luciferase activity (Fig. 1A; p < 0.001), indicating that Pu.1 can stimulate the expression of this CTSS construct. This stimulation is dose dependent, with the maximum activity achieved by transfection with 0.5 μg of the Pu.1-containing expression construct.

Both CTSS and Pu.1 are constitutively expressed at high levels in macrophages, including the mouse RAW cell line. To determine whether Pu.1 participates in CTSS expression in these cells, RAW cells were cotransfected with the CTSS promoter-reporter and DN-Pu.1 construct (Fig. 1B). Cotransfection of RAW cells with DN-Pu.1 results in statistically significant abrogation of CTSS luciferase activity. This attenuation of CTSS activity is dose dependent, with a maximum inhibition of CTSS promoter activity of −50% in this cell line. The effect of DN-Pu.1 on CTSS activity does not appear to be a nonspecific one, because promoter activity for a different gene, NOS2, was not significantly affected by cotransfection with DN-Pu.1 (Fig. 1C). These data are consistent with the proposition that Pu.1 is an important regulator of CTSS expression in professional APCs, including macrophages.

To identify the most important Pu.1 response elements in the CTSS promoter, we designed a series of promoter-reporter deletion constructs containing 5′ deletions of the CTSS promoter. Constructs were then transiently transfected into A549 cells for determination of the transcriptional response to Pu.1. Pu.1 cotransfection with the full-length CTSS (−859/+32) construct resulted in a 5- to 6-fold increase in luciferase activity (Fig. 1D), similar to the data shown in Fig. 1A. Deletions of the 5′ region progressively and gradually decreased Pu.1-dependent activation of CTSS as shorter regions were used. The shortest construct (CTSS (−75/+32)) failed to demonstrate any significant Pu.1-dependent activation. This progressive decline in CTSS activity shown in the deletion series suggests that Pu.1 may be acting at multiple sites to augment CTSS expression.

Attenuation of Pu.1 expression decreases CTSS promoter activity and expression in RAW cells

Does Pu.1 participate in regulation of the endogenous CTSS gene? To investigate this question, RAW cell lines were transfected with a sRNA construct designed to inhibit the expression of Pu.1 (Fig. 2). RAW cell lines were cloned out that do (PU.1: 4-6, 4-9, and 7-6) and do not (PU.1: 4-11) appreciably attenuate Pu.1 protein expression, as determined by immunoblot analysis (Fig. 2A). Importantly, CTSS expression at the mRNA and protein levels is attenuated in the cell lines with suppressed Pu.1 expression (PU.1: 4-9, 4-6, and 7-6) compared with the cell line that does not suppress Pu.1 expression (PU.1 4-11; Fig. 2B). Similarly, there is a decrease in active CTSS protein in the PU.1 7-6 cells, as measured...
Sequence analysis of the CTSS promoter region reveals 10 PU.1 consensus binding elements (GGAA/T) located at −48, −139, −221, −524, −567, −587, −593, −663, −771, and −795 bp 5′ from the transcriptional start site. The sites at −48 and −139 are of particular interest because they flank the ISRE located at −100 bp. Also, the sites located at −587 and −593 are intriguing, because they are in close proximity to each other, providing an enticing platform for PU.1 binding. To study which of these potential binding sites is capable of associating with PU.1, EMSA supershift experiments were performed using oligonucleotides derived from each of these sites and NE from RAW cells. Because of the close proximity of the PU.1 consensus sites at −587 and −593, a single oligonucleotide was used for both these sites in the EMSAs. Oligonucleotides derived from the CTSS promoter at positions −48, −139, and −587/−593 exhibit clear differences in migration patterns after supershifting with the PU.1 Ab, but not with control ETS1 and GATA1 Abs, suggesting that these oligonucleotides are complexed with PU.1 (Fig. 3, arrows). EMSA supershift experiments using oligonucleotides derived from the other PU.1 consensus sequences within the CTSS promoter, as listed above, do not exhibit binding (data not shown).

Mutations of PU.1 consensus sequences attenuate CTSS activation

To confirm the functional importance of these sites in mediating PU.1-induced activation of the CTSS promoter, a series of promoter-reporter constructs were generated within the full-length CTSS (−856/+32) containing mutations within the PU.1 consensus sites to abolish PU.1 binding (GGAA/T to GAGA/T). Based on the data shown in Fig. 3, mutations were generated at positions −48, −139, and −587/−593, where binding was observed, as well as at positions −663, −771, and −795, where binding was not seen. Baseline activity levels in RAW cells transfected with the CTSS promoter-reporter constructs containing point mutations at −48 and −139 were significantly lower (Fig. 4A). These data are consistent with the EMSA supershift experiments shown in Fig. 3 and indicate that these two PU.1 consensus sites play an important role in activation of the CTSS promoter. These findings also suggest the −48 and −139 sites have a dominant functional role and were of particular interest because they flanked the ISRE site located at −100 bp, which has previously been demonstrated to be important for up-regulation of CTSS promoter activity (6). Given that there was no statistically significant functional activity at the −587/−593 position, the −48 and −139 sites were the subject of further analysis.

PU.1 transcription products bind to the functionally active Ets consensus binding sites within the CTSS promoter

To confirm the interaction between PU.1 and the CTSS promoter and define the sites of interaction, EMSA studies were performed with PU.1 in vitro transcription/translation products, focusing on the functional sites (−48 and −139) in the CTSS promoter identified above (Fig. 4A). There was a specific PU.1 band for both the

FIGURE 1. PU.1 regulates CTSS promoter activity. A, A549 cells were transiently transfected with the full-length CTSS promoter-reporter construct CTSS (−859/+32) and increasing concentrations of the PU.1 expression construct. Luciferase activity was measured 48 h after transfection and was normalized to the 0-μg PU.1 sample. Each bar represents the mean ± SEM from five samples. The data shown are from one of four representative experiments. B and C, RAW cells were transiently transfected with CTSS (−859/+32) or NOS2 (−1485/+31) control promoter and the DN-PU.1 expression construct or empty control. Luciferase activity was measured 48 h after transfection. Each bar represents the mean ± SEM from three samples. The data shown are from one of three representative experiments. D, A549 cells were transiently transfected with the CTSS deletion constructs and the PU.1 expression (●) or empty (■) control vector, followed by measurement of luciferase activity. There was a progressive decline in the PU.1-induced CTSS activation as smaller promoter-reporter constructs were used, suggesting that there are several sites of activation. Each bar represents the fold induction (mean ± SEM) based on the basal activity of the full-length construct CTSS (−859/+32) from three samples. The data shown are from one of three representative experiments.
Attenuation of PU.1 expression decreases CTSS RNA and protein expression in RAW cells. A, Immunoblot of PU.1 in RAW cells lines generated by stable transfection with siRNA PU.1 constructs to attenuate PU.1 activity. Stable cell lines were generated that do (PU.1: 4-6, 4-9, and 7-6) and do not (PU.1 4-11) exhibit decreased protein levels of PU.1.

B, Northern and immunoblot analyses of mRNA and cell lysates, respectively, derived from the RAW cell lines in A, showing decreased CTSS expression in the siRNA cell lines with reduced PU.1 activity (PU.1: 4-6, 4-9, and 7-6) compared with the unaffected PU.1 4-11 line. Eight micrograms of RNA and 25 μg of total protein were loaded in each lane, respectively. The Northern blots and immunoblots were stripped and reprobed for 28S RNA to verify equal protein loading.

C, Immunoblot analysis of CTSS protein active site labeling demonstrated decreased CTSS protein levels and activity in the RAW cell line PU.1 7-6 compared with either PU.1 4-11 or wild-type (WT) cells. Twenty-five micrograms of total protein was loaded in each lane.

PU.1 associates with the endogenous CTSS promoter

PU.1 can activate several different signaling pathways within cells, some of which may have secondary effects on CTSS expression and activation. To show that at least one pathway of PU.1 activation is via direct binding of PU.1 to the endogenous CTSS promoter, we performed ChIP analysis on both PU.1-transfected human epithelial cells and a human promyelocytic cell line that constitutively expresses PU.1 (29). For the transfection experiments we chose 293T cells because the percentage of cells that are transfected in 293T cells (25–30%) is greater than that observed with A549 cells (5–10%). Similar to A549 cells, unstimulated 293T cells do not constitutively express either CTSS or PU.1. To assess the direct binding of PU.1 to the endogenous CTSS promoter in a human cell line that constitutively expresses PU.1, ChIP analysis was performed on HL-60 cells.

Based on the previous results (Fig. 4) showing that the PU.1 consensus sites located at −48 and −139 oligonucleotides that was not present when the empty vector transcription product was incubated with these oligonucleotides (Fig. 4B, arrows, lanes 2 and 3, and lanes 9 and 10). The specificity of this band was confirmed with the use of an identical competitor, which resulted in abrogated PU.1 binding (lanes 4 and 11), and nonidentical competitor in which PU.1 was bound (lanes 5 and 12). Additionally, PU.1 did not bind to radio-labeled oligonucleotides containing a mutated PU.1 binding site (Fig. 4B, lanes 7 and 14). The −48 and −139 PU.1 binding site mutations were identical with those that were shown to significantly lower CTSS promoter activation (Fig. 4A). Consistent with a specific DNA-protein interaction between PU.1 and CTSS promoter oligonucleotides, there were clear differences in the migration patterns after supershifting with the PU.1 Ab (Fig. 4B, lanes 6 and 13). This study confirms PU.1 binding to the −48 and −139 CTSS promoter oligonucleotides. The more prominent binding of PU.1 with the −139 probe compared with the −48 probe in this assay could be due to differences in binding of the oligonucleotides to PU.1 or technical differences in this in vitro assay. The preponderance of the data, including the NE EMSA (Fig. 3) and functional data (Fig. 4A), show equivalent effects at both the −48 and −139 sites. These findings with PU.1 protein, in addition to the RAW NE EMSA data, confirm a specific association between PU.1 and the CTSS promoter. These data suggest that PU.1 binds to at least two different sites within the CTSS promoter, with oligonucleotides derived from positions closer to the transcriptional start site showing a greater ability to complex with PU.1.
PU.1 Ab compared with the negative control samples. The positive control sample consisted of input DNA after reversal of the protein-DNA cross-links (Fig. 5A).

Quantitation of the PCR products by SYBR Green fluorescence spectroscopy demonstrated that there was ~23-fold more product in the transfected, PU.1-immunoprecipitated sample than

FIGURE 3. PU.1 binds to three Ets consensus elements within the CTSS promoter. EMSA experiments were performed on NE from RAW cells with oligonucleotides derived from the CTSS promoter at the indicated locations. Supershift analyses with a PU.1 Ab, but not with an ETS1 or GATA1 Ab, show clear differences in migration patterns when using oligonucleotides derived from positions −48, −139, and −587/−593 in the CTSS promoter (arrows), indicating PU.1 binding with these sequences. Differences in migration patterns in supershift EMSAs were not observed when using oligonucleotides derived from PU.1 sites located at −221, −524, −567, −663, −771, and −795 (data not shown).

FIGURE 4. Mutations of PU.1 consensus sequences attenuate CTSS activation and abrogate binding to CTSS promoter. A, RAW cells were transiently transfected with mutant CTSS promoter reporter constructs, followed by measurement of luciferase activity. Each bar represents the mean ± SEM from three samples. The data shown are from one of three representative experiments. B, EMSA performed on PU.1 transcription/translation products demonstrated a specific PU.1 band (small and large arrows, lanes 3 and 10) that was not present with transcription/translation products from the empty vector (lanes 2 and 9). This PU.1 band was abrogated with 32P-labeled −48 and −139 oligonucleotides with mutant PU.1 binding sites (MUT; lanes 7 and 14). Identical cold competition oligonucleotides (IC) also resulted in the complete loss of the specific PU.1 band that was not altered by the nonidentical competitor (NIC), (IC, lanes 4 and 11; NIC, lanes 5 and 12). There was also a clear difference in banding patterns with supershift with a PU.1 Ab, consistent with a direct interaction between PU.1 and these CTSS promoter sites (+, lanes 6 and 13). WT, Wild type.
in the negative control in 293T cells (Fig. 5B). These data show that PU.1 directly binds to the proximal CTSS promoter and support the data shown in Figs. 3 and 4 implicating this area in PU.1-dependent transcription of CTSS.

PU.1 can interact with IRF-1 and IRF-4 plus IRF-8 to promote CTSS expression

One intriguing facet of PU.1 is its ability to associate with and enhance the activity of other transcription factors, including those belonging to the IRF family (19). Given the fact that two PU.1 consensus sites at −48 and −139 flank an ISRE site within the CTSS promoter, we wanted to investigate whether PU.1 could interact with IRF family members to augment CTSS expression.

Two IRF transcription family members, IRF-4 and IRF-8, were considered because, like PU.1 and CTSS, they are constitutively expressed in APCs (30). To investigate whether PU.1 and IRF proteins can interact and augment CTSS expression, we conducted cotransfection experiments in A549 cells with PU.1 and IRF (IRF-1, IRF-2, IRF-3, IRF-4, IRF-5, IRF-6, and IRF-8) expression constructs. As shown in Fig. 6A, IRF-1 augmented CTSS expression both by itself and in conjunction with PU.1. Alternatively, cotransfection of IRF-2 with PU.1 did not significantly enhance activation (Fig. 6B). Similar negative results were observed when
using IRF-3, IRF-5, and IRF-6 expression constructs alone and in conjunction with PU.1 (data not shown). Interestingly, neither IRF-4 nor IRF-8, alone or in combination with PU.1, was able to increase CTSS expression (Fig. 6C). However, when IRF-4, IRF-8, and PU.1 constructs were all transfected together, there was increased CTSS expression beyond that observed with PU.1 alone (Fig. 6C). Although it is possible that IRF-4 and IRF-8 cooperatively may induce the expression of other genes that control the CTSS promoter, these data suggest that these three transcription factors may interact as a complex to mediate activation of this promoter. Thus, PU.1 can cooperate with several members of the IRF family to augment the expression of CTSS.

Discussion

This is the first report linking PU.1 as an important transcriptional regulator of a member of the papain-like family of cysteine proteases. Our data demonstrate that PU.1-induced activation of CTSS transcription is mediated by direct binding of PU.1 to a number of consensus sites within the proximal portion of the CTSS promoter. This enhancement of CTSS expression is further augmented by IRF family members, including two of whom have a similar expression pattern as PU.1 and CTSS (IRF-4 and IRF-8). Thus, this proximal promoter region encompassing the PU.1 sites at −48 and −139 as well as the ISRE site at −100 represents a core promoter area critical for constitutive CTSS expression in professional APCs, and inducible expression by IFN-γ (via IRF-1) in other cell types (6). These data illuminate a new pathway by which PU.1 alters the intracellular proteolytic milieu and have several important implications.

CTSS is highly expressed in professional APCs, including B cells, dendritic cells, and macrophages, and is intimately tied to MHC class II maturation and function in both human and mouse APCs (8, 9, 13). Interestingly, several recent reports show that PU.1 can induce the expression of MHC class II in B cells, mast cells, monocytes, and dendritic cells (31–34). This up-regulation of MHC class II in monocytes requires the acidic, glutamine-rich, and DNA binding domain, but not the proline-, glutamine-, serine-, and threonine-rich domain of PU.1. In contrast to CTSS, PU.1-induced enhancement of MHC class II expression occurs indirectly, at least in B cells, through activation of the CIITA promoter III (34). The CTSS promoter does not contain an X box consensus site, nor does transfection of CIITA significantly alter CTSS expression (data not shown). In an analogous fashion, IRF-1 strongly induces CTSS activity by directly binding to an ISRE in the CTSS promoter, whereas for MHC class II, IRF-1 acts more indirectly through CIITA, primarily via promoter IV (6). Thus, both the inducible and constitutive transcriptional regulation of CTSS and MHC class II are closely linked, albeit through slightly different pathways.

This manuscript adds to the growing body of literature showing that PU.1 is an important regulator of proteolytic enzyme expression in myeloid cells. PU.1 controls the expression of two serine proteases, neutrophil elastase and proteinase-3 (35, 36). These proteases are transcriptionally generated in the promyelocytic stage of neutrophil development. More recent reports show that PU.1 up-regulates expression of the aspartic protease, cathepsin E, in mouse B cells and human adenocarcinoma carcinoma cells (37, 38). Interestingly, cathepsin E activity is tied to Ag processing critical for MHC class II function in mouse microglial cells (39). PU.1-induced manipulation of the proteolytic environment in myeloid cells has important metabolic consequences, because these enzymes, CTSS included, may participate in disease processes such as atherosclerosis and emphysema (5, 40–42).

One important implication of our study is that cytokines and growth factors that modulate PU.1 levels will also affect the intracellular proteolytic milieu via CTSS expression levels. For example, macrophages deficient in MIF expression also exhibit decreased PU.1 activity (18). Indeed, naive alveolar macrophages harvested from MIF−/− mice show a 40–50% decrease in CTSS mRNA levels (data not shown). Mice deficient in the growth factor GM-CSF also exhibit very low levels of PU.1 that significantly alter macrophage functions within the lung (17). It is currently unknown whether alterations in PU.1 levels in GM-CSF−/− lung macrophages are enough to attenuate CTSS activity, or whether any of the functional abnormalities observed in these macrophages are related to decreased CTSS activity.

One notable characteristic of PU.1 is its ability to interact with IRF family members in promoting gene expression. There is a significant body of literature showing that PU.1 can associate with either IRF-4 or IRF-8 to synergistically augment the expression of genes such as human IL-1β (21). The data presented in this manuscript are slightly different in two respects. First, the CTSS promoter requires all three transcription factors expressed together to augment expression above that obtained by PU.1 alone (Fig. 6C). Most previous data suggests that PU.1 can associate with either IRF-4 or IRF-8 alone to stimulate gene activity. Importantly, all three of these transcription factors are expressed in macrophages (43, 44). Second, the CTSS promoter does not contain the classic combined PU.1/ISRE consensus site (ETS/IRF composite element) found in other promoters, including human IL-1β and human TLR-4, consisting of a PU.1 consensus site immediately 5’ to the ISRE site (45). Instead, PU.1 binding to the consensus sites flanking the ISRE at −48 and −139 may interact with the IRF family members in larger scale multiprotein complexes. The exact structural features of these complexes that regulate CTSS expression remain to be elucidated.

Several pieces of evidence indicate that PU.1 is probably one of several factors driving CTSS expression in APCs. First, the cellular expression patterns of PU.1 and CTSS, although similar, are not identical. PU.1 is expressed in neutrophils, whereas CTSS is not (15). Second, transient transfections of 293T cells with the PU.1 expression construct alone fails to increase expression of the endogenous CTSS gene, indicating that PU.1 may be necessary, but not sufficient, for CTSS expression in these cells (data not shown). Finally, the expression of CTSS at both the mRNA and protein levels is not absent in the PU.1 siRNA transfected RAW cell lines: 4-6, 4-9, and 7-6 (Fig. 2, 4-6, 4-9, and 7-6). This may be secondary to the residual PU.1 activity in this cell line or could be the result of a PU.1-independent pathway. For example, IRF-1 can stimulate CTSS expression in epithelial cells independently of PU.1 (6). These caveats notwithstanding, the data presented in this manuscript demonstrate that PU.1 plays an important role in regulation of CTSS transcription and activity.

In summary, we show that PU.1 can physically bind with and enhance CTSS promoter activity in epithelial cells. Inhibition of PU.1 in mouse macrophages abrogates CTSS expression and activity. Furthermore, several IRF family members additionally augment PU.1-dependent activation of this promoter. Together, these data suggest that PU.1 is an important regulator of CTSS, and that the expression of this protease is closely linked with the development and maturation of myeloid cell lineages. These data uncover a new pathway by which PU.1 can modulate the intracellular proteolytic environment, thereby manipulating processes mediated by this enzyme, including MHC class II-restricted Ag processing and presentation.

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