Cystatin C Antagonizes Transforming Growth Factor β Signaling in Normal and Cancer Cells

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Abstract

Cystatin C (CystC) is a secreted cysteine protease inhibitor that regulates bone resorption, neutrophil chemotaxis, and tissue inflammation, as well as resistance to bacterial and viral infections. CystC is ubiquitously expressed and present in most bodily fluids where it inhibits the activities of cathepsins, a family of cysteine proteases that can promote cancer cell invasion and metastasis. Transforming growth factor β (TGF-β) is a multifunctional cytokine endowed with both tumor-suppressing and tumor-promoting activities. We show herein that TGF-β treatment up-regulated CystC transcript and protein in murine 3T3-L1 fibroblasts. Moreover, CystC mRNA expression was down-regulated in ~50% of human malignancies, particularly cancers of the stomach, uterus, colon, and kidney. Overexpression of CystC in human HT1080 fibrosarcoma cells antagonized their invasion through synthetic basement membranes in part *via* a cathepsin-dependent pathway. Independent of effects on cathepsin activity, CystC also reduced HT1080 cell gene expression stimulated by TGF-β. Invasion of 3T3-L1 cells occurred through both cathepsin- and TGF-β-dependent pathways. Both pathways were blocked by CystC, but only the TGF-β-dependent pathway was blocked by a CystC mutant (*i.e.*, Δ14CystC) that is impaired in its ability to inhibit cathepsin activity. Moreover, CystC and Δ14CystC both inhibited 3T3-L1 cell gene expression stimulated by TGF-β. We further show that CystC antagonized TGF-β binding to its cell surface receptors, doing so by interacting physically with the TGF-β type II receptor and antagonizing its binding of TGF-β. Collectively, our findings have identified CystC as a novel TGF-β receptor antagonist, as well as a novel CystC-mediated feedback loop that inhibits TGF-β signaling.

Introduction

The cystatin superfamily of cysteine protease inhibitors is comprised of three major families: Type 1 cystatins, which are cytosolic and include stefins A and B; Type 2 cystatins, which are present in most bodily fluids and include cystatins C, D, E, F, and S; and Type 3 cystatins, which are present in plasma and include the kininogens and fetuin (1, 2). Collectively, these molecules inactivate cysteine proteases and thus regulate (*a*) bone resorption, neutrophil chemotaxis, and tissue inflammation; (*b*) hormone processing and antigen presentation; and (*c*) resistance to bacterial and viral infections (1–3). Cystatin C (CystC) is an ubiquitously expressed, small molecular weight (M_r ~16,000) secretory protein that inactivates members of the cathepsin family of cysteine proteases, the activities of which in normal tissues regulate protein catabolism, antigen presentation, bone resorption, and hormone processing, and couple to cleavage of membrane and extracellular matrix (ECM) proteins during tissue remodeling. In diseased tissues, particularly malignant and arthritic conditions, cathepsins stimulate cell migration, invasion, and metastasis [for review, see Refs. (4–7)]. Through their conserved cysteine protease inhibitor motifs, cystatins, including CystC, inactivate cathepsins by inserting a wedge-shaped hairpin loop into cathepsin active-site clefts, thus forming reversible, high-affinity enzyme-inhibitor complexes (8–10). Independent of its effects on cathepsin activity, CystC also regulates cell proliferation (11, 12), raising the possibility that CystC targets protease-dependent and -independent pathways.

Mutations in or altered expression of CystC has been linked to the development and progression of several human pathologies. For instance, a single point mutation in CystC causes Hereditary CystC Amyloid Angiopathy, a lethal autosomal dominant disease that results in massive cerebral hemorrhages in early adulthood (13). Moreover, altered sera CystC concentration has been proposed as a diagnostic marker for glomerular filtration rate and kidney function (14, 15), while altered sera CystC or cathepsin B:CystC complex concentrations were suggested as diagnostic and prognostic indicators for cancers of the skin, colon, and lung (3, 16–18). Thus, altered CystC concentrations within cell microenvironments have dire consequences to the development and progression of human diseases.

Transforming growth factor β (TGF-β) is a multifunctional cytokine that governs cell growth and motility in part through its regulation of cell microenvironments, and thus plays a prominent role in regulating disease development in humans (19–21). Critical to regulation of cell microenvironments by TGF-β is its induction or repression of cytokines, growth factors, and ECM proteins by fibroblasts (19). Herein we show that TGF-β stimulates CystC transcript and protein expression in murine 3T3-L1 fibroblasts. We further show that CystC mRNA was aberrantly down-regulated in ~50% of human tumors, and that its overexpression in highly malignant human HT1080 fibrosarcoma cells reduced their invasion in

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part via a cathepsin-dependent manner. Interestingly, CystC also inhibited HT1080 cell expression of TGF-β-responsive genes independent of its effect on cathepsin activity. Invasion of 3T3-L1 cells proceeds through cathepsin- and TGF-β-dependent pathways: CystC inhibited both pathways, while a CystC mutant (*i.e.*, Δ14CystC) impaired its ability to antagonize cathepsin activity selectively inhibited TGF-β-dependent invasion, suggesting that CystC targets protease-dependent and -independent signaling pathways. Accordingly, both CystC and Δ14CystC significantly reduced TGF-β-stimulated gene expression in 3T3-L1 cells. We further show that CystC antagonized TGF-β binding to its cell surface receptors, doing so by interacting physically with the TGF-β type II receptor (TβR-II) and preventing its binding of TGF-β. Collectively, our findings identify a novel CystC-mediated feedback loop that inhibits TGF-β signaling in normal and cancer cells, as well as establish CystC as a novel TGF-β receptor antagonist.

Results

TGF-β1 Induces CystC Expression in 3T3-L1 Cells

TGF-β governs cell microenvironments by regulating fibroblast expression and secretion of cytokines, growth factors, and ECM proteins that alter the survival, proliferation, and motility of normal and cancer cells (19). To identify fibroblast secretory proteins whose expression is regulated by TGF-β, we collected, concentrated, and fractionated by 2D-electrophoresis proteins present in naïve- and TGF-β-conditioned media of murine 3T3-L1 fibroblasts. Fractionated proteins were immobilized to Immobilon-P, and were visualized by coomassie staining and autoradiography. Differentially expressed proteins regulated by TGF-β were excised and subjected to Edman sequencing. As shown in Fig. 1A, a highly basic protein of M_r ~18,000 was prominently induced by TGF-β. Edman sequencing of this protein returned an amino acid sequence of (NH2)-ATPKQGPR-(COOH), corresponding to residues 21–28 of murine CystC.

TGF-β previously has been shown to stimulate CystC transcript expression in murine embryo cells (22–24). To confirm that CystC expression was indeed induced in 3T3-L1 cells by TGF-β and to establish the mechanism for this effect, we performed Northern blot analysis on total RNA isolated from TGF-β-treated 3T3-L1 cells. As shown in Fig. 1B, TGF-β stimulated 3T3-L1 cells to synthesize CystC transcript in a time-dependent manner. Collectively, these findings establish CystC as a novel gene target for TGF-β in 3T3-L1 cells, ultimately leading to increased production and secretion of CystC protein.

Tumorigenesis Alters CystC Expression in Human Tissues

Altered CystC expression has been associated with the cancer development and progression (3, 16–18, 25–28). To identify human cancers potentially susceptible to altered CystC expression, we hybridized a radiolabeled human CystC cDNA probe to a membrane arrayed with matched normal/tumor cDNAs generated from cancer patients. Of the 68 patients surveyed, CystC expression was altered in 49% (33/68) of the tumors, of which 91% (30/33) showed down-regulation (Fig. 2). Significantly attenuated CystC expression was especially evident in cancers of the stomach (75%; 6/8 cases), uterus (71%; 5/7 cases), prostate (67%; 2/3 cases), colon (55%; 6/11 cases), and kidney (47%; 7/15 cases; Fig. 2). In contrast to previous reports (3, 16–18), we found CystC expression to be up-regulated significantly in only 9% of human tumors that exhibited altered expression of CystC (Fig. 2). Thus, while tumorigenesis can promote enhanced CystC expression, our findings indicate that tumorigenesis primarily suppresses CystC expression, supporting the notion that it normally functions to suppress tumor formation, as well as the processes of invasion and metastasis.

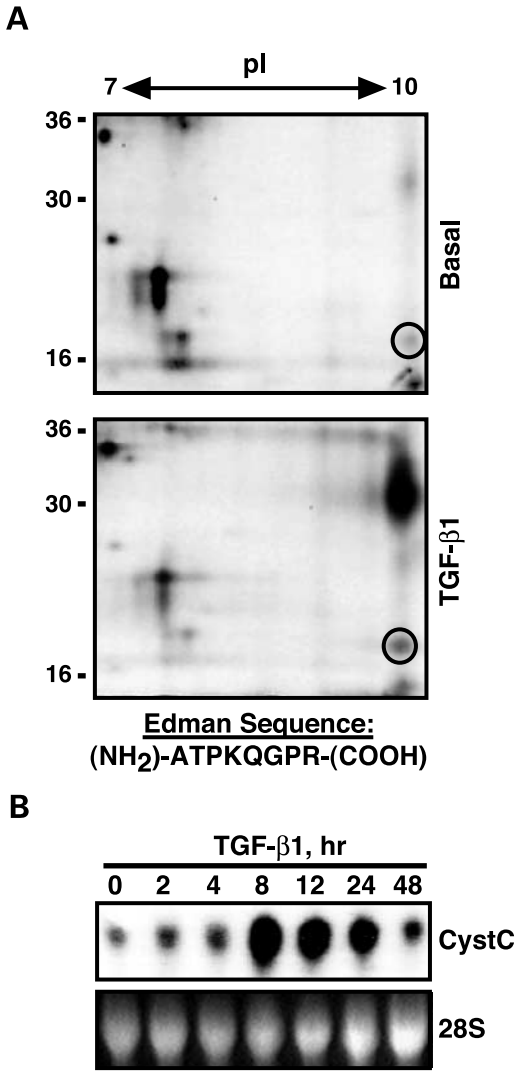

FIGURE 1. TGF-β induces CystC expression in 3T3-L1 cells. **A.** Metabolically labeled naïve or TGF-β-conditioned 3T3-L1 cell media were collected, concentrated, and fractionated by 2D-electrophoresis before electrophoretic transfer to Immobilon-P. Proteins were visualized by Coomassie blue staining and audioradiography; those proteins whose Expression was regulated by TGF-β were excised and subjected to Edman sequencing. *Circled region* shows murine CystC. **B.** Total RNA (10 μg/lane) prepared from TGF-β1-treated 3T3-L1 cells was hybridized with a radiolabeled human CystC cDNA probe (*upper panel*). The uniformity of mRNA loading was monitored by ethidium bromide staining to visualize the 28S rRNA (*lower panel*).

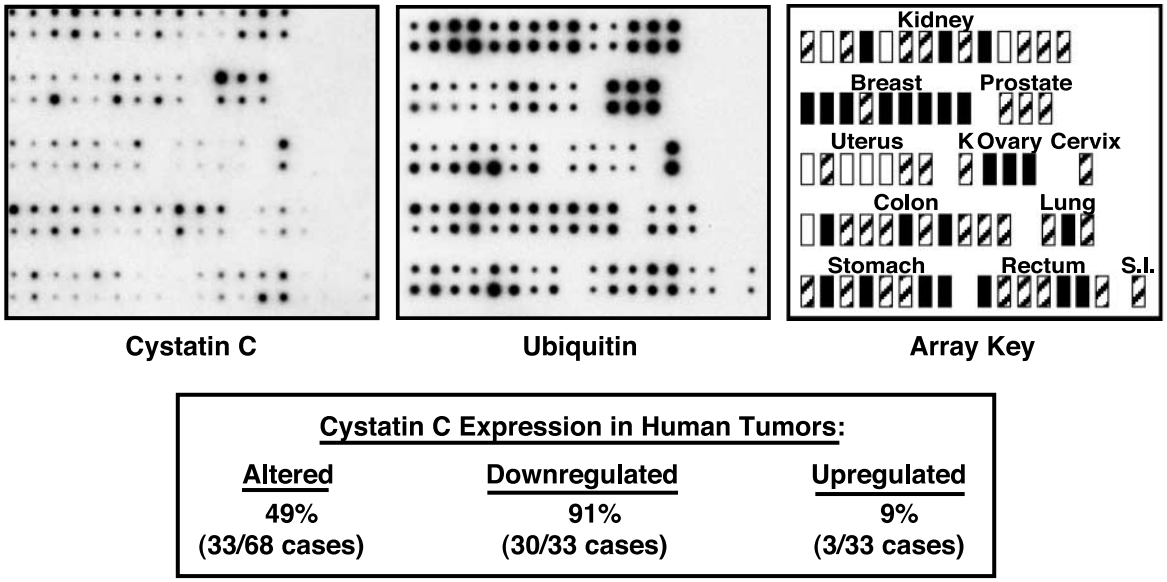

FIGURE 2. Tumorigenesis alters CystC expression in human tissues. Radiolabeled cDNA probes corresponding to either human CystC (*left panel*) or ubiquitin (*middle panel*) were hybridized to match human normal/tumor cDNA array. Shown are the resulting autoradiographs depicting CystC and ubiquitin expression in paired normal (*upper spot*) and malignant (*bottom spot*) tissues. CystC expression was normalized to that of ubiquitin and tumor:normal tissue CystC expression ratios were determined. Ratios ≥ 2 or ≤ 0.5 were considered significant. Tumor type and metastasis status are indicated by: (*a*) *open boxes,* no information; (*b*) *filled boxes,* metastasis observed; and (*c*) *stripped boxes,* metastasis not observed (*right panel*). *K,* kidney; *S.I.,* small intestine.

CystC Inhibits Cathepsin-Mediated Invasion in HT1080 Cells

Cathepsins are normally lysosomal cysteine proteases that function in intracellular protein catabolism, as well as in bone resorption, hormone activation, and antigen processing (4–7). During tumorigenesis, cancer cells typically up-regulate cathepsin expression, particularly cathepsins B, D, and L (4), via several mechanisms, including gene amplification, elevated mRNA transcription and splicing, and alternative promoter activation (5, 7). The net effect of these genetic alterations results in enhanced cathepsin protein production and, consequently, in the redistribution of cathepsins from lysosomes to plasma membranes, or to the extracellular milieu following their secretion, whereupon they localize to the leading edge of invasive tumors during induction of cancer cell invasion and metastasis (29–32).

Recent evidence implicates cell type-specific roles for intracellular and extracellular cathepsins (*e.g.*, cathepsin B) in promoting cancer cell invasion (31, 33, 34). Human HT1080 fibrosarcoma cells are highly tumorigenic and invasive: they also express large quantities of secreted cathepsin B and comparably little CystC (35). However, the contribution of intracellular *versus* extracellular cathepsin activity in mediating HT1080 cell invasion is not yet known. To distinguish between these possibilities, we infected HT1080 cells that stably express the murine ecotropic receptor (36) with control (*i.e.*, GFP) or CystC retrovirus. HT1080 cells also were infected with Δ14CystC retrovirus, which encodes for a CystC deletion mutant lacking the entire cysteine protease inhibitor signature (*i.e.*, residues 80–93) located in the first hairpin loop. Point mutations (37) or deletions (38) within the first hairpin loop of chicken cystatin dramatically reduce its ability to inactivate cathepsins *in vitro* (*e.g.*, 50–10,000-fold lower K_i values). We therefore reasoned that abrogating the entire cysteine protease inhibitor signature would similarly impair the ability of CystC to inhibit cathepsin activity. We further reasoned that if extracellular cathepsin activity mediates HT1080 cell invasion, then overexpression of secreted CystC would inhibit their invasion through synthetic basement membranes, while Δ14CystC would be without effect.

Figure 3A shows that cathepsin B-mediated proteolysis of laminin was completely inhibited by recombinant CystC, but only minimally so by recombinant Δ14CystC, demonstrating that the cathepsin-inhibiting activity of CystC was severely impaired by removal of the cystatin protease inhibitor signature. As shown in Fig. 3B, HT1080 cells infected with CystC and Δ14CystC retrovirus expressed and secreted recombinant CystC proteins into the media, while those infected with control (*i.e.*, GFP) retrovirus were negative for recombinant CystC expression. In accord with our hypothesis, CystC expression significantly inhibited HT1080 cell invasion through Matrigel matrices (Fig. 3C). In contrast, expression of Δ14CystC failed to affect HT1080 cell invasion (Fig. 3C). Furthermore, treating HT1080 cells with the cell-impermeable cathepsin B inhibitor II (Calbiochem, La Jolla, CA) significantly reduced their invasion through Matrigel matrices (by $47.1 \pm 8.5\%$; $n = 3$, $P < 0.05$), while treatment with the cell-permeable cathepsin B inhibitor, CA-074ME (Calbiochem), failed to effect their invasiveness ($101.0 \pm 5.6\%$ of control; $n = 3$). Although we cannot definitively exclude the possibility that cathepsin B inhibitor II and CystC are both internalized via an endocytic pathway and thus are acting on intracellular cathepsins, our findings support the conclusion that HT1080 cell invasion occurs in part via cathepsin protease activities localized extracellularly, not intracellularly.

CystC Inhibits TGF-β-Responsive Reporter Gene Expression via a Cathepsin-Independent Mechanism in HT1080 Cells

During tumorigenesis, TGF-β is frequently converted from a suppressor to a promoter of cancer cell growth, invasion, and metastasis (19, 20). TGF-β stimulation of HT1080 cells had no effect on their invasion through Matrigel matrices, nor on their expression of CystC (data not shown). Thus, while TGF-β clearly regulates CystC expression in murine embryo (22), vascular smooth muscle (24), uterine decidual (23), and 3T3-L1 (Fig. 1) cells, the coupling of TGF-β to CystC expression in HT1080 cells appears dysregulated. CystC has also been reported to stimulate DNA synthesis in normal and transformed murine Swiss 3T3 fibroblasts (11), and in rat mesangial cells (12), as well as inhibit melanoma cell motility (39). However, overexpression of CystC or Δ14CystC in HT1080 cells failed to effect their synthesis of DNA and migration to fibronectin (data not shown).

Extracellular cathepsin expression [*e.g.*, cathepsin B; (40–42)] has been linked to the activation of latent TGF-β from inactive ECM depots. We therefore hypothesized that CystC expression might impact TGF-β signaling via a cathepsin-dependent mechanism. To test this hypothesis, we measured changes in luciferase expression driven by the synthetic p3TP-luciferase reporter gene (43) in control- and CystC-expressing HT1080 cells. As shown in Fig. 3D, CystC significantly inhibited TGF-β-stimulated luciferase activity driven by the p3TP promoter. Surprisingly, Δ14CystC was equally effective as CystC in inhibiting luciferase activity stimulated by TGF-β (Fig. 3D). Collectively, these findings identify CystC as a novel antagonist of TGF-β signaling, doing so through a cathepsin-independent pathway.

CystC Inhibits Tonic and TGF-β-Stimulated Invasion in 3T3-L1 Cells

CystC previously has been reported to regulate cell proliferation (11, 12) and motility (39). Similar to HT1080 cells, we found that CystC failed to effect 3T3-L1 cell DNA synthesis and migration to fibronectin (data not shown). However, we have found that 3T3-L1 fibroblasts readily invade through Matrigel matrices, and that TGF-β enhances their ability to do so (see below). To determine the effects of CystC on the invasiveness of 3T3-L1 cells, we generated stable polyclonal populations of 3T3-L1 cells expressing GFP, CystC, or Δ14CystC by bicistronic retroviral infection. As shown in Fig. 4A, the resulting 3T3-L1 cell lines had purities ≥90% and expressed GFP indistinguishably. Furthermore, 3T3-L1 cells infected with CystC or Δ14CystC retroviruses

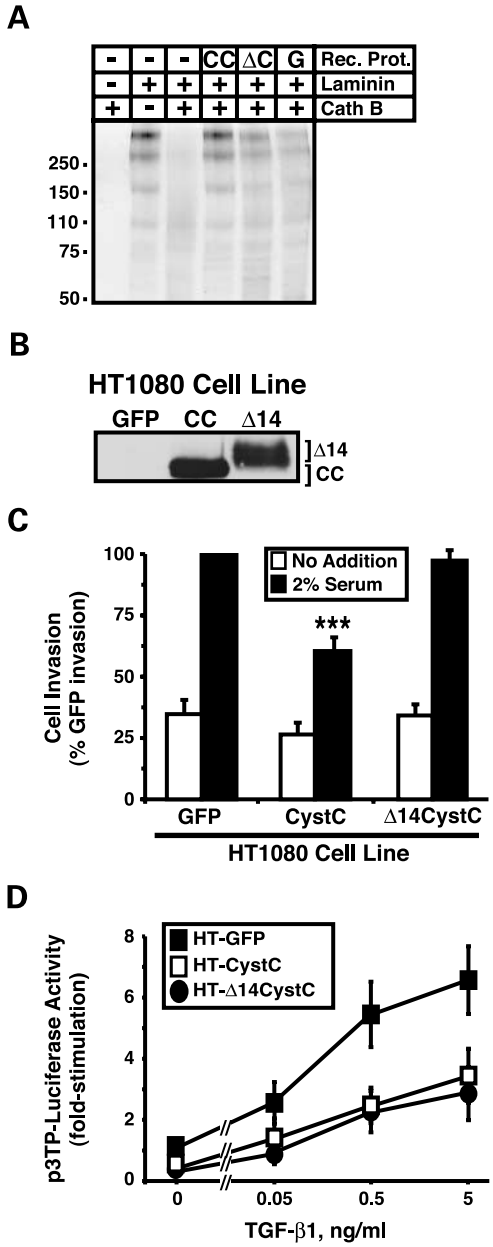

FIGURE 3. CystC inhibits cathepsin-mediated invasion and TGF-β-responsive reporter gene expression in HT1080 cells. **A.** DTT/EDTA-activated human cathepsin B (2 μg; Sigma, St. Louis, MO) was incubated with murine laminin (10 μg; Sigma) in the absence or presence of 10 μg/reaction of either recombinant glutathione *S*-transferase (GST)-CystC (*CC*), GST-Δ14CystC (Δ*C*), or GST (*G*) for 5 h at 37°C. The reaction mixtures were fractionated through 7.5% SDS-PAGE before Coomassie staining of the gel to visualize laminin. Shown is a representative Coomassie-stained gel from an experiment that was repeated once with identical results. **B.** Conditioned media of HT1080 cells infected with control (*i.e.*, GFP), CystC, or Δ14CystC retroviruses were collected and immunoblotted with anti-CystC antibodies. HT1080 cells transduced with CystC (*CC*) or Δ14CystC (Δ*14*) retroviruses constitutively secreted recombinant CystC protein into the medium. **C.** GFP-, CystC-, or Δ14CystC-expressing HT1080 cells were allowed to invade through Matrigel-coated membranes for 48 h. *Columns,* means of three (Δ*14CystC*) and nine (*GFP* and *CystC*) independent experiments presented as the percentage invasion relative to GFP-expressing HT1080 cells; *bars,* SE. CystC expression, but not that of Δ14CystC, significantly inhibited HT1080 cell invasion (***, $P < 0.05$; Student's *t* test). **D.** GFP-, CystC-, or Δ14CystC-expressing HT1080 cells were transiently transfected with p3TP-luciferase cDNA and pCMV-β-gal cDNA. The transfectants were stimulated with increasing concentrations of TGF-β1 (0 → 5 ng/ml) as indicated, and subsequently processed to measure luciferase and β-gal activities. *Points,* means of luciferase activities of four independent experiments normalized to untreated GFP-expressing cells; *bars,* SE.

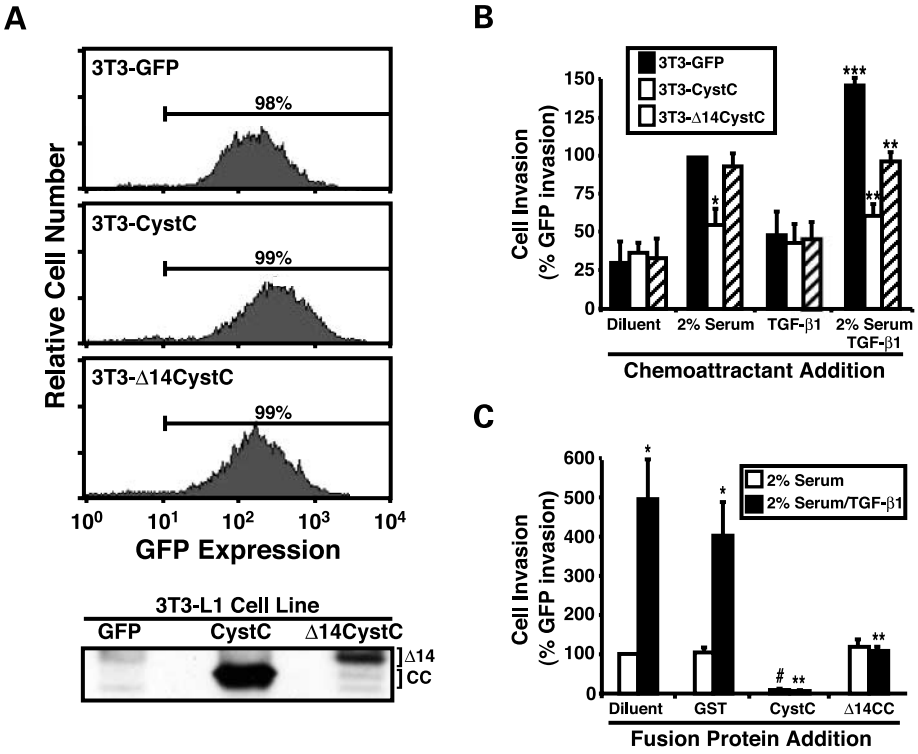

FIGURE 4. CystC inhibits tonic and TGF-β-stimulated invasion in 3T3-L1 cells. **A.** Murine 3T3-L1 cells were infected with ecotropic retrovirus encoding either GFP (*i.e.*, control), CystC, or Δ14CystC. The infectants were FACS-sorted by GFP expression (highest 10%) to yield stable polyclonal populations of control (*top panel*), CystC (*middle panel*), and Δ14CystC-expressing (*bottom panel*) 3T3-L1 cells having equivalent GFP expression levels at a positivity rate of ≥90%. Immunoblotting 3T3-L1 cell conditioned media with anti-CystC antibodies demonstrated that 3T3-L1 cells transduced with CystC- or Δ14CystC-retroviruses constitutively secreted recombinant CystC proteins into the medium. **B.** GFP-, CystC-, or Δ14CystC-expressing 3T3-L1 cells were allowed to invade through Matrigel-coated filters in the absence or presence of TGF-β1 (5 ng/ml) for 48 h. *Columns,* means of four independent experiments presented as the percentage invasion relative to GFP-expressing 3T3-L1 cells; *bars,* SE. TGF-β1 significantly enhanced 3T3-L1 cell invasion (***, $P < 0.05$; Student's *t* test). CystC expression significantly inhibited tonic (*, $P < 0.05$; Student's *t* test) and TGF-β1-stimulated (**, $P < 0.05$; Student's *t* test) 3T3-L1 cell invasion, while cell invasion, while Δ14CystC expression only significantly inhibited TGF-β-stimulated 3T3-L1 cell invasion (**, $P < 0.05$; Student's *t* test). **C.** Control or TGF-β1-stimulated (5 ng/ml) 3T3-L1 cells were allowed to invade through Matrigel-coated filters for 48 h in the absence or presence of recombinant (10 μg/ml) GST, GST-CystC, or GST-Δ14CystC as indicated. *Columns,* means of three independent experiments presented as the percentage invasion relative to untreated 3T3-L1 cells; *bars,* SE. TGF-β1 significantly enhanced 3T3-L1 cell invasion (*, $P < 0.05$; Student's *t* test). Recombinant CystC significantly inhibited tonic (#, $P < 0.05$; Student's *t* test) and TGF-β1-stimulated (**, $P < 0.05$; Student's *t* test) 3T3-L1 cell invasion, while Δ14CystC expression only significantly inhibited TGF-β-stimulated 3T3-L1 cell invasion (**, $P < 0.05$; Student's *t* test).

expressed and secreted high levels of recombinant CystC protein into the media, while those infected with control retrovirus (*i.e.,* GFP) were negative for recombinant CystC expression (Fig. 4A).

As expected, 3T3-L1 cells readily invaded through Matrigel matrices when stimulated by serum: this response was unaffected by Δ14CystC, but was inhibited significantly by CystC (Fig. 4B). Figure 4B also shows that 3T3-L1 cells treated with TGF-β exhibited a trend toward enhanced invasion; however, TGF-β treatment in combination with serum induced significantly more 3T3-L1 cell invasion than that by serum alone (Fig. 4B). Interestingly, CystC expression blocked both components of 3T3-L1 cell invasion, whereas Δ14CystC blocked only the TGF-β-dependent component (Fig. 4B). Moreover, the inhibitory effects of CystC on 3T3-L1 cell invasion could be recapitulated by addition of recombinant CystC fusion proteins. As shown in Fig. 4C, treatment of 3T3-L1 cells with recombinant GST (10 μg/ml) failed to effect their invasion induced by serum or serum:TGF-β. In contrast, recombinant CystC administration blocked 3T3-L1 cell invasion stimulated by serum and serum:TGF-β, whereas recombinant Δ14CystC selectively blocked that by TGF-β (Fig. 4C). Collectively, these findings indicate that 3T3-L1 cell invasion proceeds through cathepsin- and TGF-β-dependent pathways. Moreover, our findings show that CystC abrogated both pathways, while Δ14CystC blocked only the TGF-β-stimulated pathway.

CystC and ΔCystC Inhibit TGF-β-Stimulated Reporter Gene Expression in 3T3-L1 Cells

Our findings that TGF-β signaling was inhibited by CystC (Figs. 3 and 4) independent of its actions on cathepsin led us to hypothesize CystC as a general antagonist of TGF-β signaling. To test this hypothesis, we monitored changes in p3TP-driven luciferase activity in control, CystC-, Δ14CystC-expressing cells before and after their stimulation with TGF-β. Figure 5A shows that expression of either CystC or Δ14CystC significantly reduced TGF-β-stimulated luciferase activity as compared to control cells. Similar to their effects on 3T3-L1 cell invasion, recombinant CystC and Δ14CystC both significantly inhibited

luciferase activity stimulated by TGF-β in 3T3-L1 cells (Fig. 5B). Taken together, our findings identify CystC as a novel antagonist of TGF-β signaling, doing so through a cathepsin-independent mechanism.

CystC Antagonizes TGF-β1 Binding to TGF-β Receptors

Our findings thus far have identified CystC as a novel TGF-β antagonist (Figs. 3–5). The ability of recombinant CystC (and Δ14CystC) to recapitulate the inhibitory effects of CystC expression on TGF-β signaling led us to speculate that CystC inhibits TGF-β signaling by antagonizing the interaction between TGF-β and its receptors. This hypothesis seemed especially attractive given the fact that the Type 3 cystatin family member, fetuin (also known as α2-HS-glycoprotein), inhibits TGF-β signaling by physically interacting with and preventing the binding of TGF-β to its receptors (44–46). Although co-immunoprecipitation and affinity pull-down assays both failed to demonstrate a direct physical interaction between CystC and TGF-β1 (data not shown), iodinated TGF-β1 binding and cross-linking assays showed that recombinant CystC dose-dependently inhibited the binding of TGF-β to its cell surface receptors (Fig. 6). The reduction in TGF-β1 binding was specific to CystC because increasing concentrations of recombinant Jun(1-79), a GST fusion protein of similar size and purified in parallel with GST-CystC, had no effect on the binding of iodinated TGF-β1 to its receptors (Fig. 6, *middle panel*). Taken together, this finding demonstrates that one mechanism whereby CystC inhibits TGF-β signaling is by preventing the binding of TGF-β to its cell surface receptors, thus implicating CystC as a novel TGF-β receptor antagonist.

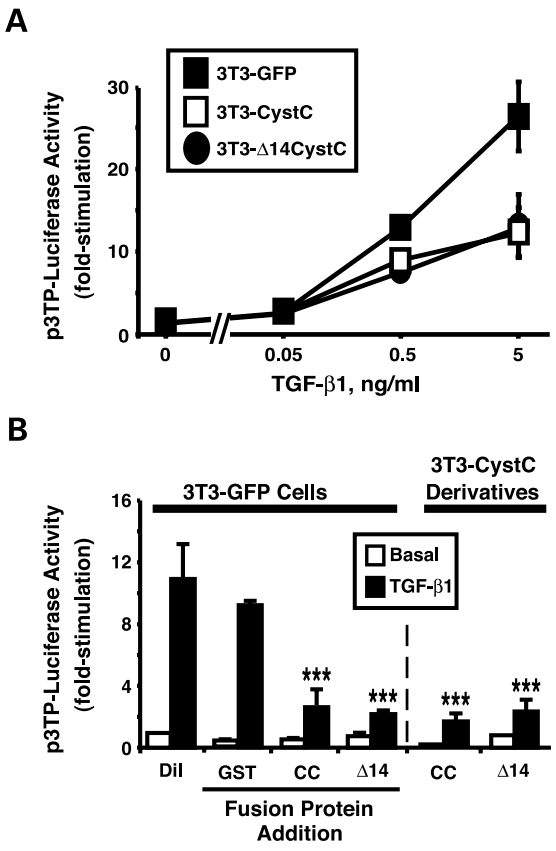

FIGURE 5. CystC and Δ14CystC inhibit TGF-β-stimulated reporter gene expression in 3T3-L1 cells. **A.** GFP-, CystC-, or Δ14CystC-expressing 3T3-L1 cells were transiently transfected with p3TP-luciferase cDNA and pCMV-β-gal cDNA and subsequently stimulated with increasing concentrations of TGF-β1 (0 → 5 ng/ml) as indicated. Afterward, luciferase and β-gal activities contained in detergent-solubilized cell extracts were measured. *Points,* mean luciferase activities of four independent experiments normalized to untreated GFP-expressing cells; *bars,* SE. **B.** GFP-, CystC-, or Δ14CystC-expressing 3T3-L1 cells were transiently transfected with p3TP-luciferase and pCMV-β-gal plasmids as above. The transfectants were stimulated with TGF-β1 (5 ng/ml) in the absence or presence of recombinant (10 μg/ml) GST, GST-CystC, or GST-Δ14CystC as indicated. *Columns,* mean luciferase activities of five independent experiments normalized to untreated GFP-expressing cells; *bars,* SE. CystC and Δ14CystC significantly inhibited TGF-β-stimulated luciferase activity driven by the synthetic p3TP promoter (***, $P < 0.05$; Student's *t* test).

CystC Inhibits TGF-β1 Binding to TβR-II by Interacting Physically With TβR-II

Transmembrane signaling by TGF-β commences by its binding to either TβR-III, which then associates with and binds to TβR-II, or directly to TβR-II, which then associates with and binds to the TβR-I (19, 47). Figure 6 shows that CystC equally reduced the binding of TGF-β1 to all three of its cell surface receptors, which suggests that CystC selectively antagonized initial TGF-β binding, not its subsequent receptor multimerization. Given the low abundance of TβR-III expressed in Mv1Lu cells (Fig. 6), we hypothesized that CystC antagonized TGF-β signaling by preventing TGF-β binding to TβR-II. To test this hypothesis, we performed an *in vitro* TGF-β capture assay that measured the effects of CystC on the binding of iodinated TGF-β1 to soluble human TβR-II produced by human 293T cells transiently transfected with sTβR-II cDNA. This sTβR-II construct was chosen for study because it binds TGF-β (48) and has itself been used to antagonize TGF-β signaling (49–51). As expected, significantly more iodinated TGF-β1 was captured by 293T cell conditioned media containing sTβR-II than was captured by control media (Fig. 7A). More importantly, recombinant CystC completely abrogated the binding of iodinated TGF-β1 to sTβR-II (Fig. 7A). Thus, CystC inhibits TGF-β signaling by antagonizing TβR-II binding of TGF-β.

In an attempt to establish the mechanism for the inhibitory actions of CystC on TβR-II binding of TGF-β, we performed an *in vitro* TβR-II retention assay that measured the ability of CystC to capture sTβR-II from conditioned media of 293T cells transiently transfected with sTβR-II cDNA. As shown in Fig. 7B, recombinant GST failed to capture sTβR-II from conditioned media of 293T cells expressing sTβR-II. In stark contrast, sTβR-II protein was readily captured from 293T cell conditioned media by recombinant CystC. Collectively, our findings have identified CystC as a novel TGF-β receptor antagonist, doing so by interacting physically with TβR-II and preventing its binding of TGF-β.

Discussion

Cell microenvironments play an important role in regulating the physiology and homeostasis of cells, including their survival, proliferation, differentiation, and motility (52). TGF-β is

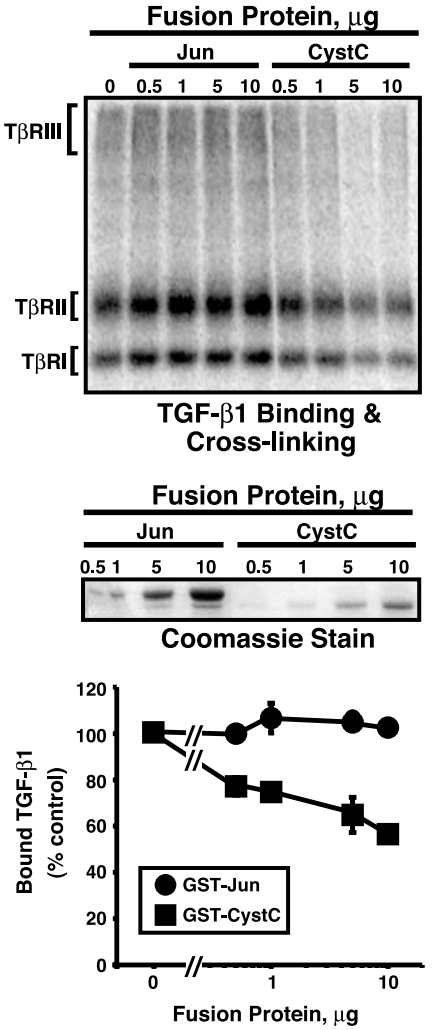

FIGURE 6. CystC antagonizes TGF-β1 binding to TGF-β receptors. Mink lung Mv1Lu epithelial cells were incubated for 3 h at 4°C with [^{125}I]TGF-β1 (200 pM) in the absence or presence of increasing concentrations (0 → 10 μg/ml) of recombinant GST-Jun(1-79) or GST-CystC as indicated. Cytokine:receptor complexes were cross-linked by addition of disuccinimidyl suberate and subsequently isolated from detergent-solubilized whole cell extracts by immunoprecipitation with anti-TβR-II antibodies. Data are a representative phosphor image of iodinated TGF-β1 bound extensively to TβR-I and TβR-II and less extensively to TβR-III. *Middle panel* shows the Coomassie staining of increasing concentrations of purified GST-Jun(1-79) and GST-CystC. The *accompanying graph* depicts the mean TGF-β1 (±SE) binding observed in six independent experiments and is presented as the percentage of TGF-β1 binding normalized to that in the absence of added fusion protein.

a powerful tumor suppressor that prevents cancer development by inhibiting cell cycle progression, and by creating cell microenvironments that inhibit uncontrolled cell growth, invasion, and metastasis. During cancer progression, the tumor suppressing function of TGF-β is frequently subverted, thus transforming TGF-β from a suppressor of cancer formation to a promoter of its growth and metastasis (19, 20). Although mutations in the TGF-β signaling system occur during carcinogenesis and contribute to tumor formation by abrogating TGF-β-mediated cell cycle arrest (19, 20), these aberrances do not explain the paradoxical function of TGF-β to promote the proliferation, invasion, and metastasis of cancer cells previously liberated from its growth inhibitory actions. Alternatively, recent studies have established fibroblasts as instrumental intermediaries of tumor growth and metastasis stimulated by TGF-β. When activated by TGF-β, fibroblasts synthesize and secrete a variety of cytokines, growth factors, and ECM proteins into tumor:host microenvironments. These secretory proteins facilitate tumor:host cross-talk that ultimately promotes the induction, selection, and expansion of neoplastic cells by enhancing their growth, survival, and motility (53). We therefore sought to identify these TGF-β-regulated fibroblast secretory proteins, and to determine their function in normal and cancerous cells.

We now present CystC as a novel gene target for TGF-β in 3T3-L1 fibroblasts. We further show that CystC expression is down-regulated in ~50% of human cancers (Fig. 2), and conversely, that augmenting CystC expression in highly malignant fibrosarcoma cells significantly reduced their invasion through synthetic basement membranes (Fig. 3). Most strikingly, our study identified CystC as a novel antagonist of TGF-β signaling (Figs. 3–5). Indeed, we show for the first time that CystC inhibits gene expression (Figs. 3 and 5) and cellular invasion (Fig. 5) stimulated by TGF-β. We further show that CystC antagonizes TGF-β binding to its cell surface receptors (Fig. 6), doing so by interacting physically with TβR-II and abrogating its binding of TGF-β (Fig. 7). Thus, our study defines a novel cathepsin-independent function for CystC, and has potentially identified a novel CystC-mediated feedback loop designed to inhibit TGF-β signaling.

The inactivation of cathepsin protease activity by CystC has been a focal point of numerous investigations aimed at understanding the invasive properties of cancer cells (29, 54, 55). Although it is generally accepted that extracellular cathepsin mediates cancer cell invasion, recent evidence also implicates a cell type-specific role for intracellular cathepsins in mediating ECM degradation and cancer cell invasion (31, 33, 34). With respect to highly tumorigenic and invasive HT1080 cells, we show that CystC expression, but not that of Δ14CystC, significantly inhibited their invasion through Matrigel matrices. Moreover, the cell-permeable cathepsin B inhibitor, CA-074ME, failed to alter HT1080 cell invasion, whereas its cell impermeable counterpart, cathepsin B inhibitor II, fully recapitulated the inhibitory effect of CystC on HT1080 cell invasion. At present, we cannot exclude the possibility that cathepsin B inhibitor II and secreted CystC are both internalized via an endocytic pathway and thus are acting on intracellular cathepsins. However, the complete ineffectiveness of CA-074ME to alter HT1080 cell invasion suggests that intracellular cathepsin B activity does not play a significant role in promoting the invasion of HT1080 cells. In addition, the broad-spectrum inhibitory activities of CystC and cathepsin B inhibitor II do not permit us to ascertain the identities of extracellular cathepsins operant in mediating HT1080 cell invasion. Nevertheless, our findings do demonstrate that the invasion of HT1080 cells occurs in part via the actions of extracellular cathepsins, the activities of which are subject to inactivation by CystC.

Our study also provides the first evidence that 3T3-L1 cell invasion proceeds through a bifurcated signaling system

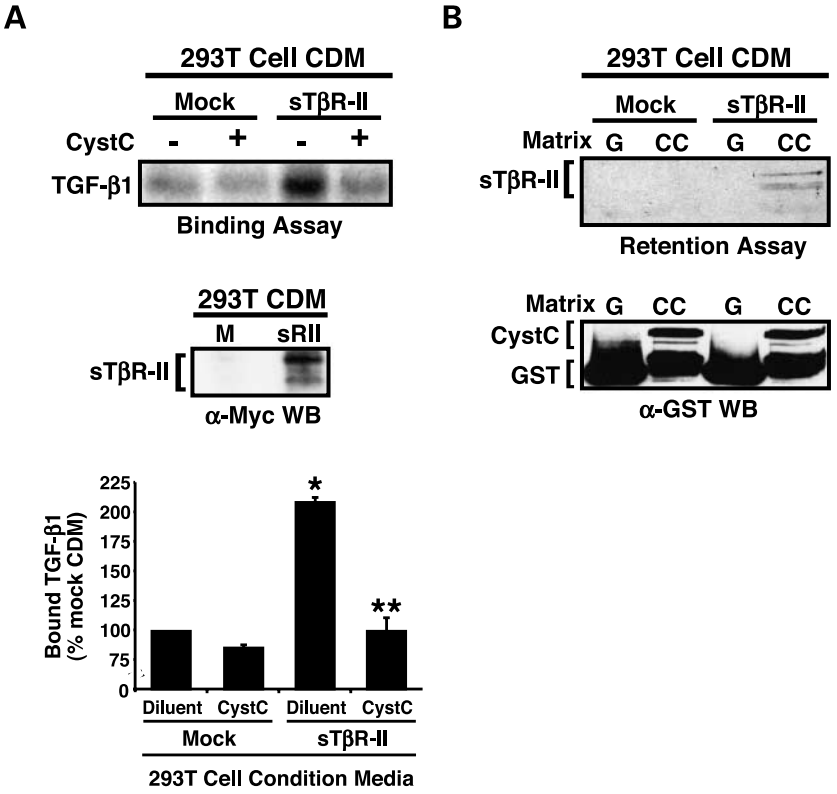

FIGURE 7. CystC inhibits TGF-β1 binding to TβR-II by interacting physically with TβR-II. **A.** Human 293T cells were transiently transfected with empty vector or sTβR-II cDNA, which encodes for a soluble, secreted protein comprised of the ligand binding extracellular domain of TβR-II. Conditioned media from mock (M)- or sTβR-II-transfected 293T cells were collected and incubated for 2 h at 4°C with [^{125}I]TGF-β1 (200 pM) in the absence or presence of GST-CystC (10 μg/ml) as indicated. Cytokine:receptor complexes were isolated by immunoprecipitation with anti-Myc antibodies. *Upper panel* shows a representative autoradiograph of iodinated TGF-β1 bound to sTβR-II, while the *middle panel* demonstrates sTβR-II expression detected by Myc immunoreactivity. *Lower panel* depicts the mean TGF-β1 (±SE) binding from three independent experiments presented as the percentage of TGF-β1 binding normalized to conditioned media of mock-transfected cells without added fusion protein. sTβR-II significantly enhanced TGF-β1 capture from conditioned media (*, $P < 0.05$; Student's *t* test). The binding of TGF-β1 to sTβR-II was inhibited significantly by recombinant CystC (**, $P < 0.05$; Student's *t* test). **B.** Human 293T cells were transiently transfected with empty vector or sTβR-II cDNA as above. Conditioned media from mock- or sTβR-II-transfected cells were collected and applied to columns comprised of GST (*G*) or CystC (*CC*). After extensive PBS washing, bound proteins were eluted with Laemmli sample buffer and fractionated through 12% SDS-PAGE before their immobilization to nitrocellulose. Retention of sTβR-II was detected by immunoblotting the membrane with anti-Myc antibodies. Differences in column protein loading were monitored by reprobing the stripped membrane with HRP-conjugated anti-GST antibodies. Data are from a representative experiment that was repeated twice with similar results.

coupled to cathepsin activity and TGF-β signaling. Moreover, the differential abilities of CystC (*i.e.*, both pathways) and Δ14CystC (*i.e.*, TGF-β pathway) to inhibit 3T3-L1 cell invasion implicates CystC as key regulator not only of cathepsin-mediated invasion, but also that mediated by TGF-β. Although the identity of additional proteolytic pathways targeted by TGF-β in 3T3-L1 cells remains unknown, we suspect involvement of members of the matrix metalloproteinase family (*e.g.*, MMP-2 and MMP-9), the expression and activity of which are regulated by TGF-β (21). Another candidate system is likely the urokinase plasminogen activator system (uPA), the expression and activity of which are up-regulated in metastatic human malignancies, leading to enhanced ECM degradation and growth factor activation, and to increased cancer cell migration, invasion, and angiogenesis (56). Recently, Sloane and colleagues (32, 57) proposed caveolae as proteolytic organizing centers that bring together cathepsin B, uPA, plasminogen, and MMPs to the invasive face of cancer cells. Future studies exploiting the anti-TGF-β properties of Δ14CystC may prove useful in dissecting the interplay between these invasive pathways and TGF-β during tumorigenesis, and in preventing the oncogenic properties of TGF-β that arise during cancer progression.

It is also noteworthy that cathepsin B (29–31) and TGF-β (58) both localize to the invading face of malignant tumors. In addition to its role in cancer cell invasion (4–7), cathepsin B also mediates latent TGF-β activation (40–42). Thus, up-regulated and/or dysregulated cathepsin B activity observed in malignant cells would be predicted to enhance the oncogenic effects of TGF-β. On the basis of our findings, we propose that measures designed to deliver CystC to developing tumors will reduce their tumorigenicity by inhibiting (*a*) cathepsin-mediated invasion and metastasis, (*b*) cathepsin B-mediated activation of latent TGF-β from inactive ECM depots, and (*c*) TβR-II binding of TGF-β and, consequently, TGF-β signal transduction.

We (Fig. 2) and others (3, 16–18, 25–28) have observed aberrant CystC expression elicited by tumorigenesis. Although

it is clear that augmenting CystC expression in cancer cells negatively impacts their invasiveness *in vitro* [Fig. 3; (39, 54, 55)] and tumorigenicity *in vivo* (54), it is unclear why the sera of patients with melanoma (59) or colorectal (16) cancers contain more CystC as compared to normal volunteers. Interestingly, CystC-deficient mice are viable and exhibit no pronounced abnormalities (60). Moreover, no differences in the latency and growth of s.c. and intracerebral tumors were detected in wild-type and CystC-null mice. However, lung colonization by cancer cells was suppressed in CystC-deficient mice as compared to their wild-type counterparts (60). Although the mechanism mediating this paradoxical effect of CystC on cancer cell metastasis is currently unknown, the authors speculate that dysregulated cathepsin B activity suppresses metastasis by catabolizing local cytokines/growth factors necessary for metastatic cancer cell seeding and growth (60). On the basis of our findings, we would instead propose that CystC deficiency increases tonic TGF-β signaling, resulting in enhanced tumor suppression and the creation of cell microenvironments that fail to efficiently support the metastatic spread and seeding of cancer cells. Future studies clearly need to test this hypothesis, as well as determine whether CystC deficiency potentiates cellular response to TGF-β (*i.e.*, loss of CystC-mediated feedback loop).

Finally, the Type 3 cystatin family member, fetuin, has also been characterized as a TGF-β antagonist (44–46). Fetuin contains a 19-amino-acid TRH1 domain [*T*GF-β *r*eceptor II *h*omology 1; (46)] that interacts physically with TGF-β and prevents its binding to TGF-β receptors (46). Although CystC contains COOH-terminal 21-amino-acid sequence having similarity to TRH1, we were unable to observe a direct physical interaction between TGF-β and CystC. In contrast, we found that CystC inhibits TGF-β signaling by interacting physically with TβR-II, thus preventing its binding of TGF-β both in live cells and *in vitro*. Interestingly, preliminary structure-function studies have implicated the COOH-terminal domain of CystC in mediating its antagonism of TGF-β signaling.[2] Given the identification of two CystC superfamily members capable of inhibiting TGF-β signaling, it is tempting to speculate that additional CystC superfamily members will function similarly to antagonize the activities of other TGF-β superfamily members, as well as the activities of other cytokines/growth factors. Importantly, our findings give credence to future studies aimed at exploiting the anti-TGF-β properties of CystC (or Δ14CystC) to selectively inhibit the oncogenic activities of TGF-β. In support of this endeavor, we recently determined that CystC and Δ14CystC both abrogate the ability of TGF-β to stimulate morphological transformation in rat NRK fibroblasts and epithelial-to-mesenchymal transdifferentiation in murine NMuMG mammary epithelial cells.[2] Indeed, these findings may form the basis for rational drug design to facilitate the development of specific TGF-β receptor antagonists necessary to improve the therapeutic response of human malignancies, as well as a variety of proliferative and fibrotic diseases regulated by TGF-β.

[2] J.P. Sokol and W.P. Schiemann, unpublished findings.

Materials and Methods

Human CystC Plasmids

A retroviral CystC vector was synthesized by PCR amplifying the full-length human CystC cDNA from EST 4183311. The resulting PCR product was shuttled through the pcDNA3.1/Myc-His B vector (Invitrogen, Carlsbad, CA) at *Eco*RI (NH_2 terminus) and *Xho*I (COOH terminus) restriction sites to COOH-terminally tag human CystC with Myc- and $(His)_6$-tags. Afterward, the resulting tagged CystC cDNA was PCR amplified using oligonucleotides containing *Eco*RI (NH_2 terminus) and *Xho*I (COOH terminus) restriction sites, and subsequently ligated into identical sites immediately upstream of the IRES in the bicistronic retroviral vector, pMSCV-IRES-GFP (61, 62).

A retroviral vector encoding a CystC molecule that lacked amino acids 80–93 (termed Δ14CystC) was constructed by PCR amplifying the pGEX-4T1-contained Δ14CystC cDNA insert (see below), which was shuttled through the pSecTag B vector (Invitrogen) at *Nco*I (NH_2 terminus) and *Bgl*II (COOH terminus) restriction sites. This cloning step COOH-terminally tagged the Δ14CystC cDNA with the Myc- and $(His)_6$-tags, as well as appended the Igκ leader sequence to its NH_2 terminus to permit its secretion when expressed in mammalian cells. The resulting tagged Δ14CystC was PCR amplified using oligonucleotides containing *Hpa*I (NH_2 terminus) and *Eco*RI (COOH terminus) restriction sites that facilitated ligation of the PCR fragment into pMSCV-IRES-GFP. When expressed in mammalian cells, Δ14CystC protein was slightly larger than that of its corresponding wild-type CystC due to additional NH_2-terminal amino acids appended to Δ14CystC by the pSecTag vector.

All CystC and Δ14CystC cDNA inserts were sequenced in their entirety on an Applied Biosystems 377A DNA sequencing machine.

Fusion Protein Construction and Purification

A CystC fusion protein was synthesized by PCR amplifying full-length human CystC cDNA (less its signal sequence) using oligonucleotides containing *Eco*RI (NH_2 terminus) and *Xho*I (COOH terminus) restriction sites. The resulting PCR fragment was appended to the COOH terminus of GST encoded by the bacterial expression vector, pGEX-4T1 (Amersham Pharmacia Biotech, Piscataway, NJ). Site-directed mutagenesis of GST-CystC to delete 14 amino acids located within the conserved cysteine protease inhibitor motif (residues 80–93; termed Δ14CystC) was performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. All fusion cDNA inserts were sequenced in their entirety on an Applied Biosystems 377A DNA sequencing machine.

The expression and purification of various GST fusion proteins from transformed *Escherichia coli* was as described previously (63).

Soluble TβR-II Plasmid

A soluble human TβR-II was synthesized by PCR amplifying the extracellular domain of TβR-II (sTβR-II; nucleotides 72–516) using oligonucleotides containing *Kpn*I

(NH_2 terminus) and *Not*I (COOH terminus) restriction sites. The resulting PCR product was ligated into corresponding sites in the pSecTag B vector, which COOH-terminally tagged the sTβR-II cDNA with Myc- and $(His)_6$-tags and appended the Igκ leader sequence at its NH_2 terminus. The resulting sTβR-II was sequenced in its entirety on an Applied Biosystems 377A DNA sequencing machine.

Isolation and Identification of Murine CystC

Murine 3T3-L1 fibroblasts were cultured in 15-cm plates in DMEM supplemented with 10% fetal bovine serum (FBS). On attaining 90% confluency, the cells were washed extensively in PBS and then rendered quiescent by incubation in serum-free DMEM for 12 h at 37°C. Quiescent 3T3-L1 cells were metabolically labeled with [^{35}S]methionine in the absence or presence of TGF-β1 (10 ng/ml) for 12 h at 37°C. Afterward, naïve- and TGF-β-conditioned media were collected (10 plates/condition), clarified by centrifugation, and concentrated initially to ~3 ml in an Amicon concentrator (1000 Da MWC), and ultimately via trichloroacetic acid/deoxycholate precipitation. The resulting protein pellets were resuspended, neutralized, and precipitated a second time with acetone before their resuspension in isoelectric focusing buffer (8 M urea, 4% CHAPS, 10 mM DTT, and 0.2% 3–10 ampholytes). Protein samples were applied to 11 cm isoelectric focusing strips (pH 3–10) and developed according to the manufacturer's recommendations (three strips/condition; Amersham Pharmacia Biotech). The isoelectric focused proteins then were fractionated through 10% SDS-PAGE, and subsequently transferred electrophoretically to Immobilon-P (Millipore, Bradford, MA). Secreted proteins were visualized by coomassie staining, and by autoradiography of the dried membranes. A differentially expressed M_r ~18,000 protein that was evident in TGF-β-conditioned media was excised from the membranes and sequenced using the Sequelon-AA sequencing kit (ABI, Foster City, CA).

Northern Blotting

Quiescent 3T3-L1 cells were incubated in the absence or presence of TGF-β1 (5 ng/ml) for 0–48 h, and subsequently were harvested in RNAzol Reagent (Tel-Test, Friendswood, TX) to isolate total RNA. Ten micrograms of total RNA were electrophoresed through 1.2% agarose/formaldehyde gels and transferred to nylon membrane. The immobilized RNA was probed with a ^{32}P-labeled full-length human CystC cDNA probe for 1 h at 68°C in ExpressHyb (Clontech, Palo Alto, CA). Afterward, the membrane was washed according to the manufacturer's instructions, and CystC mRNA was visualized by autoradiography.

Tumor Array

The effects of tumorigenesis on CystC expression were examined by hybridizing a ^{32}P-labeled full-length human CystC cDNA probe to a matched human normal/tumor cDNA microarray according to the manufacturer's instructions (Clontech). CystC expression in normal and malignant human tissues was visualized by autoradiography. The cDNA microarray was stripped and reprobed with a ^{32}P-labeled ubiquitin cDNA probe provided by the manufacturer. CystC and ubiquitin cDNA signal intensities were measured using NIH Image software (version 1.62). Differential CystC expression in normal and malignant tissues was identified as follows. First, the average CystC cDNA signal intensity was calculated by summing all microarray cDNA signals and dividing this aggregate by the total number of cDNAs present in the microarray. This process was repeated to calculate average ubiquitin cDNA signal intensity. Second, individual cDNA signals were divided by their corresponding microarray average cDNA signal to yield individual cDNA signal ratios. Third, individual CystC cDNA signal ratios were divided by their corresponding to ubiquitin cDNA signal ratio to yield corrected individual cDNA signal ratios. Finally, the overall ratios of tumor CystC cDNA *versus* normal tissue CystC cDNA signal intensity were calculated by dividing a given corrected individual tumor cDNA signal ratio by its corresponding corrected normal tissue cDNA signal ratio. Overall ratios of ≥ 2 or ≤ 0.5 were considered significant.

Retroviral Infections

Control (*i.e.*, pMSCV-IRES-GFP), CystC, or Δ14CystC retroviral supernatants were produced by EcoPac2 retroviral packaging cells (Clontech) and used to infect murine 3T3-L1 fibroblasts and human HT1080 fibrosarcoma cells (grown in DMEM with 10% FBS) as described previously (61, 62). Forty-eight hours post-infection, the highest 10% of GFP-expressing cells were collected on a MoFlo cell sorter (Cytomation, Fort Collins, CO), and subsequently expanded to yield stable populations of control-, CystC-, or Δ14CystC-expressing cells having equivalent GFP levels at a positivity rate of >90%.

Western Blotting

Conditioned media (2 ml) of HT1080 or 3T3-L1 cells stably expressing GFP, CystC or Δ14CystC were collected, clarified by centrifugation, and concentrated by trichloroacetic acid/deoxycholate precipitation. The proteins were fractionated through 12% SDS-PAGE gel and subsequently transferred electrophoretically to nitrocellulose. The membrane was probed with anti-CystC polyclonal antibodies (1:1000, Upstate, Charlottesville, VA), and the resulting immunocomplexes were visualized by enhanced chemiluminescence.

Invasion Assays

The effect of CystC and Δ14CystC on the invasion of HT1080 and 3T3-L1 cells was determined as described previously (61). Briefly, upper chambers were coated with 100 μl of diluted Matrigel (1:50 in serum-free media), which was evaporated to dryness overnight at room temperature. The following morning, the Matrigel mixtures were rehydrated and subsequently cultured with control-, CystC-, or Δ14CystC-expressing HT1080 or 3T3-L1 cells at a density of 100,000 cells/chamber. Cellular invasion was stimulated by addition of 2% serum to the lower chambers. Forty-eight hours later, the cells were washed twice in ice-cold PBS and immediately fixed for 15 min with 95% ethanol. Cells remaining in

the upper chambers were removed with a cotton swab, whereas those remaining in the lower chamber were stained with crystal violet. Quantifying invading cells was determined through two independent measures which yielded identical results: (*a*) manual counting under a light microscope, and (*b*) crystal violet dye extraction by incubation of the membranes in 10% acetic acid, followed by spectrophotometry at 590 nm.

In some experiments, the effect of recombinant GST-CystC and GST-Δ14CystC on 3T3-L1 cell invasion was examined. To do so, 3T3-L1 cells (100,000 cells/chamber) were allowed to invade through Matrigel in the absence or presence of 10 μg/ml of recombinant GST, GST-CystC, or GST-Δ14CystC, together with or without 5 ng/ml of TGF-β1. All subsequent procedures were performed as described above.

Luciferase Reporter Gene Assays

Analysis of luciferase activity driven by the synthetic p3TP reporter [(43); generously provided by Dr. Joan Massague, Sloan Kettering] was performed as described previously (61). Briefly, control-, CystC-, or Δ14CystC-expressing 3T3-L1 or HT1080 cells were cultured onto 24-well plates at a density of 45,000 cells/well and allowed to adhere overnight. The cells were transiently transfected the following morning by overnight exposure to LT1 liposomes (Mirus) containing 300 ng/well of p3TP-luciferase cDNA and 100 ng/well of pCMV-β-gal cDNA, which was used to control for differences in transfection efficiency. Afterward, the cells were washed twice with PBS and stimulated overnight in serum-free media with increasing concentrations of TGF-β1 as indicated. The following morning, luciferase and β-gal activities contained in detergent-solubilized cell extracts were determined.

In some experiments, the effect of recombinant GST-CystC and GST-Δ14CystC on p3TP-luciferase activity 3T3-L1 cells was determined. To do so, 3T3-L1 cells were transiently transfected as above, and subsequently stimulated with TGF-β1 (5 ng/ml) in the absence or presence of 10 μg/ml of recombinant GST, GST-CystC, or GST-Δ14CystC. All subsequent procedures were performed as described above.

Iodinated TGF-β1 Radioligand Binding and Cross-Linking Assay

Mink lung Mv1Lu epithelial cells were plated onto six-well plates and grown in DMEM containing 10% FBS until reaching 90% confluency. The radioligand binding and cross-linking of iodinated TGF-β1 (200 pM) to Mv1Lu cells in the absence or presence of increasing concentrations of recombinant GST-Jun(1-79) or GST-CystC was performed as described previously (64). Afterward, cytokine:receptor complexes contained in detergent-solubilized whole cell extracts were isolated by immunoprecipitation with anti-TβR-II antibodies as described previously (64). TGF-β1 bound to cell surface TβR-I, TβR-II, and TβR-III was visualized by exposure of the dried gels to a phosphor screen, which was developed 1–3 days later on a Molecular Dynamics Typhoon Scanner. Total bound TGF-β1 was defined as the sum of signal intensities for TGF-β1 cross-linked to TβR-I, TβR-II, and TβR-III in each condition.

In Vitro Iodinated TGF-β1 Binding Assay

Human kidney 293T cells were plated onto six-well plates and allowed to adhere overnight in DMEM supplemented with 10% FBS. The cells were transiently transfected the following morning by overnight exposure to LT1 liposomes containing 1 μg/well sTβR-II cDNA, which encodes for the extracellular domain of TβR-II that is Myc-His-tagged at its COOH terminus. The following morning, the cells were placed in serum-free DMEM for an additional 24 h, whereupon the resulting conditioned media were collected and cleared of cellular debris by microcentrifugation. The clarified supernatants then were supplemented with [^{125}I]TGF-β1, together with or without recombinant CystC (10 μg/ml), and subsequently were tumbled for 2 h at 4°C. Afterward, TGF-β1:sTβR-II complexes were isolated by immunoprecipitation with monoclonal anti-Myc antibodies (1 μg/ml; Covance, Princeton, NJ) and fractionated through 12% SDS-PAGE. TGF-β1 bound to recombinant sTβR-II was visualized by a 1- to 3-day exposure of the dried gels to a phosphor screen, which ultimately was developed on a Molecular Dynamics Typhoon Scanner.

sTβR-II Retention Assay

Human 293T cells were cultured onto six-well plates and allowed to adhere overnight. The following morning, duplicate wells of cells were transiently transfected with 1 μg/well of either sTβR-II cDNA or pBluescript cDNA as above. Afterward, the cells were placed in 1 ml of serum-free DMEM for an additional 24 h before collecting the conditioned media, which were clarified by microcentrifugation. One milliliter of clarified supernatant was applied to columns (50–75 μl bed volumes) containing 10 μg of either GST or GST-CystC. Bound proteins were washed with 1.5 ml of PBS, and subsequently were eluted by addition of 100 μl of warmed 1× Laemmli sample buffer. Eluted proteins were fractionated through 12% SDS-PAGE and transferred electrophoretically to nitrocellulose. Retained sTβR-II was detected by probing the membrane with anti-Myc antibodies (1 μg/ml), while GST and GST-CystC were detected by reprobing the stripped membrane with horseradish peroxidase-conjugated monoclonal anti-GST antibodies (1/1000; Santa Cruz Biotechnology, Santa Cruz, CA).

Acknowledgments

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