Regulation of Clusterin Gene Expression by Transforming Growth Factor β*

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Transforming growth factor β (TGFβ) induces the expression of a wide variety of genes in many cell types. Our previous studies have shown that TGFβ stimulates both clusterin mRNA and protein levels, and induces its accumulation in the nucleus of CCL64 cells. To further investigate the molecular mechanism of clusterin mRNA induction by TGFβ, we created a 1.3-kilobase rat clusterin promoter/luciferase reporter construct. We demonstrate that TGFβ enhances luciferase activity 2.5–6-fold in transient transfection assays of epithelial, endothelial, and fibroblast cell lines. Deletional analysis reveals that an AP-1-binding site (5'-TGAGGCA) in the minimal promoter region is necessary for initiating transactivation by TGFβ. A single T to G base mutation in the AP-1 site (5'-TGAGGCA) abolishes TGFβ-induced clusterin promoter transactivation. In transcription factor decoy experiments, 23-mer oligonucleotides of wild type AP-1 reduce TGFβ induction of clusterin mRNA levels and promoter transactivation, while an oligonucleotide containing the mutated AP-1 site has no effect. Two specific protein kinase C inhibitors, GF109203X and calphostin C, block TGFβ-induced clusterin mRNA levels and promoter transactivation. Together these results indicate that TGFβ regulates clusterin gene expression through an AP-1 site and its cognate transcription factor AP-1, and requires the involvement of protein kinase C.

The transforming growth factor-β (TGFβ) family of cytokines consists of multifunctional proteins which play important roles in regulating cell growth, development, and differentiation (1–6). A number of structural and metabolic proteins, such as fibronectin and its receptor, collagen, collagenase, plasminogen activator type-1, contain consensus sequences in their regulatory region, and their cognate cis-elements (2, 12–14). Recent studies have shown that Smad proteins, which are postulated to function as TGFβ receptor-regulated transcription factors, may act as cellular mediators in TGFβ signaling of mammalian cells and play a critical role in transmitting the TGFβ signal to the nucleus (15, 16). Protein kinase C and other protein kinases have also been implicated in TGFβ-mediated regulation of gene expression. These kinases may participate in recruitment of transcription factors, such as activator protein 1 (AP-1), to modulate TGFβ-responsive gene expression (6, 7, 15, 17–22). Many TGFβ-responsive genes, such as plasminogen activator inhibitor type-1, contain AP-1 consensus sequences in their regulatory region, and the sequence is required for TGFβ regulation of genes in both growth-stimulated and growth-inhibited cell lines (7, 8, 23–26). Sp1 has been shown to participate in the regulation of human α2(I)-collagen and p21/Waf1/Cip1 gene expression by TGFβ (27, 28). Nuclear factor 1 also appears to be involved in expression of several genes regulated by TGFβ (29). TGFβ modulates interaction of these transcription factors and their cognate elements in a coordinated manner to specifically regulate TGFβ-responsive gene expression. However, the signaling pathway(s) through which TGFβ modulates gene responses in mammalian cells remains largely unknown.

The clusterin protein was first discovered in ram rete testis fluid as an ~80-kDa heterodimeric glycoprotein that facilitated the aggregation of a variety of cells in culture (30). A number of homologues of clusterin have been identified in several species (31). Clusterin is present in almost all mammalian body fluids and can also be induced or constitutively expressed in almost all cell types (30, 31). The protein has been implicated in a variety of biological processes including lipid transport, inhibition of complement attack, sperm maturation, epithelial cell differentiation, and membrane remodeling during apoptosis and implantation (31–37). Analysis of the 5'-regulatory region of the clusterin gene has revealed TGFβ inhibitory elements as well as AP-1, Sp1, and AP-2 regulatory elements in the quail, rat, and human clusterin genes (38–41). These elements are postulated to be responsible for the modulation of clusterin gene expression observed during cell differentiation, development, and embryogenesis (31, 35, 39–41). It has also been demonstrated that clusterin gene expression can be regulated by TGFβ in a cell type-dependent manner (35, 42). For example, TGFβ down-regulates clusterin mRNA levels in porcine smooth muscle cells (35), induces its gene expression in rat astrocytes in the presence of oligodendrocytes and microglia, while repressing its message in monotypic cultures of astrocytes (42). However, the mechanism(s) of regulation of clusterin gene expression by TGFβ is unknown.

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The abbreviations used are: TGFβ, transforming growth factor β; CCL64 cells, mink lung epithelial cells; BAEC, bovine aortic endothelial cell; PMMA, polymethylmethacrylate; clusterin, also known as apolipoprotein J; CDTA, 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid; AP-1, activator protein 1; DMEM, Dulbecco’s modified Eagle’s medium; PKC, protein kinase C.

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We have previously reported that TGFβ induces expression of clusterin protein and rapidly stimulates clusterin mRNA levels in a mink lung epithelial cell line CCL64 cells (MvLlu). We have also demonstrated that TGFβ selectively induces accumulation of clusterin protein in the nucleus (11, 43). To further dissect the mechanism of TGFβ-mediated regulation of clusterin gene expression, we have analyzed the intracellular signaling pathway through which TGFβ modulates gene expression. Our results demonstrate that TGFβ regulates clusterin gene expression in epithelial, fibroblasts, and in a primary culture of bovine aortic endothelial cells, at least in part, at the transcriptional level. An AP-1 site in the rat clusterin 5′-promoter region is responsible for the regulation of clusterin gene expression by TGFβ, and the effect of TGFβ on clusterin gene expression requires the involvement of protein kinase C.

MATERIALS AND METHODS

Cell Culture—Mink lung epithelial cells, HeLa cells, 3TP cells (derived from HT1080 (44), and 10T1/2 cells were cultured in DMEM/F-12 medium containing 10% newborn calf serum (Atlanta Biological Inc.) at 37 °C with 5% CO₂. The primary culture of bovine aortic endothelial cells (BEC) was grown in DMEM/F-12 medium containing 10% fetal bovine serum (Sigma) at 37 °C with 5% CO₂. The B9 hybridoma was maintained in RPMI medium containing 10% newborn calf serum at 37 °C until 70–80% confluence. The hybridoma cell line was subcloned by limited dilution and plated at 1000 cells/well and incubated in DMEM/F-12 medium, supplemented with 2 m M L-glutamine, 2 m M HEPES, 2 m M sodium pyruvate, 1 m M nonessential amino acids, and 100 U/ml penicillin, 100 μg/ml streptomycin. The cells were incubated at 37 °C for 5 h before adding TGFβ. For transfection assays with other cell lines, 20 μl of liposome reagent, 1 μg of reporter plasmid DNA, and 0.2 μg of pRL-CMV plasmid (Promega) as an internal control were used. All other procedures were the same as for transfections of CCL64 cells. After removing the transfection medium, serum or serum-free medium containing TGFβ was added to the cells and incubated for 16–20 h at 37 °C prior to the assay.

Decoy Experiments of Transcription Factors—Sense and antisense 23-mer oligonucleotides, containing either AP-1-binding site (AP-1) or its mutant (T2G), were synthesized according to phosphoramidite oligonucleotide synthesis (42) and subcloned in pGEM plasmid (Promega) as an internal control plasmid. For decoy assays analyzed by Northern blot, 20 nmol of DNA was mixed with 30 μl of liposome reagent in 2 ml of serum-free medium, and incubated at room temperature for 30 min to form a liposome-DNA complex. Cells, 80% confluent, were washed with serum-free medium prior to the addition of the liposome-DNA complex. A liposome-DNA complex was stimulated with 5 ng/ml TGFβ for 10 h and subjected to RNA extraction and Northern blot analysis. To perform decoy experiments in transient transfection assays, 0.7 μg of oligonucleotide was added along with pRL (100:1 molar ratio) into liposome reagent for transfection and luciferase activity.

Luciferase and Galactosidase Activity Assays—Following transient transfection and TGFβ stimulation, cells were washed with phosphate-buffered saline two times and lysed at room temperature in 300 μl of lysis buffer (25 mM Tris phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM CTDA, 10% glycerol, 1% Triton X-100). The cell lysate was scraped into a microcentrifuge tube and centrifuged at high speed for 30 s at room temperature to remove cell debris. Cell lysates (10 μl) were mixed with 100 μl of luciferase substrate buffer (Promega) and luciferase activity was measured immediately (M2210 Microlight and Dynatech Laboratories). For lysates from cells transfected with pRL-CMV plasmid as an internal control construct, the reporter plasmid and control plasmid luciferase activities were measured with Promega’s Dual-Luciferase Reporter Assay System according to manufacture’s protocol. To measure galactosidase activity, 150 μl of 2 μ CTDA, 2 μ phospho-molybdate, and 1 μl of 0.1% nitroblue tetrazolium, 1.33 mg/ml nitrophenyl β-D-galactopyranoside was added to 150 μl of cell lysate. Following incubation at 37 °C overnight, 600 μl of H₂O was added to β-galactosidase reaction mixture and A₄₂₀ was read with a spectrophotometer (UV160U, Shimadzu) to quantify β-galactosidase activity. The promoter/reporter luciferase activity was normalized to β-galactosidase activity or renilla luciferase activity of the Dual-Luciferase Reporter Assay System, respectively.

RESULTS

TGFβ Induces Clusterin mRNA Synthesis—We have previously demonstrated that TGFβ induces clusterin protein synthesis and accumulation of clusterin mRNA in the nuclei of CCL64 cells (11). However, the TGFβ signaling pathway mediating this induction remains unknown. Therefore, we were interested in studying the regulation of clusterin gene expression by TGFβ in an attempt to identify some of the signaling components involved. Northern blot analysis showed that TGFβ in-
Periods. As shown in Fig. 1, treatment with 5 ng/ml TGFβ induces clusterin mRNA as early as 30 min after TGFβ treatment and continues about 24 h in rapidly growing, asynchronous CCL64 cells (Fig. 1, A and B). To determine whether the induction of clusterin RNA synthesis was a consequence of the growth-inhibitory effect of TGFβ on the cells, CCL64 cells were serum starved in serum-free DMEM/F-12 medium for 24 h, prior to treatment with TGFβ, for different periods. As shown in Fig. 1C, TGFβ also induces clusterin mRNA synthesis in serum-starved, quiescent cells, indicating that induction of clusterin mRNA by TGFβ is not a result of growth inhibition of the cells. The induction of clusterin mRNA by TGFβ is not restricted to mink lung epithelial cells. The data presented in Fig. 1D demonstrates that TGFβ can also induce clusterin gene expression in other cell types. In two fibroblasts cell lines, 10T1/2 and 3TP, two epithelial cell lines, CCL64 and HeLa, and in primary BAEC, TGFβ induces clusterin mRNA levels following a 16-h treatment.

To determine whether the induction of clusterin mRNA by TGFβ is at the transcriptional level, we performed transient transfection assays in these different cell lines with a luciferase reporter gene (pRAL) containing the 1.3-kilobase 5′-regulatory region of rat clusterin gene (Fig. 2B). Following transfection, cells were treated with 5 ng/ml TGFβ for 16 or 20 h. The data (Fig. 2A and Fig. 4) show that TGFβ induces promoter transactivation with a 2.5–6-fold elevation in luciferase activity in the five cell types analyzed. In transient transfection assays of CCL64 cells, we used this same reporter construct lacking the TATA box in the regulatory region as a negative control (Fig. 2A). This construct failed to induce luciferase activity in response to TGFβ. The results provide supportive evidence that TGFβ induces clusterin gene expression, at least in part, at the transcriptional level.

Deletional Analysis of a Rat Clusterin Gene 5′-Promoter Region—To analyze the TGFβ-responsive cis-element(s) of the clusterin promoter, we constructed a series of 5′-deletions of the promoter region of rat clusterin gene. Since the 1.3-kilobase promoter region contains Sp1, AP-2, and AP-1 consensus sites, we decided to make deletions containing different combinations of these conventional binding sites as the first step in our promoter analysis (Figs. 2 and 3). The pRAL-M/Nsi deletion with 586 base pairs truncated from the 5′-promoter region, which still retains all three binding sites, is able to induce TGFβ transactivation. Two other deletions, pRAL-E/E, which remove the AP-1-binding site, and pRAL-E/E, which lack Sp1 and both Sp1 and AP-2 sites, respectively, still show TGFβ induction of promoter transactivation similar to the full-length promoter/reporter construct. This suggests that Sp1 and AP-2 are not necessary for clusterin transactivation by TGFβ. However, deletions pRAL-E/E, pRAL-p2p4, and pRAL-AP−, which remove the AP-1-binding site in the promoter region, abolished TGFβ-induced promoter transactivation (Fig. 3). The data indicate that the AP-1-binding site in the promoter region is essential for TGFβ-mediated regulation of clusterin gene expression.

The AP-1 Site Is Required for TGFβ-mediated Induction of Clusterin Gene Transcription—While the deletional analyses...
revealed that the AP-1-binding site in the clusterin promoter region is important for TGFβ-mediated induction of clusterin gene expression, we needed to determine whether the AP-1 site is the exclusive element in the clusterin promoter responsible for TGFβ-mediated gene regulation. To test this, we created a single base mutation within the AP-1-binding site of the promoter region. The wild-type AP-1-binding site is 5'-TGAGTCA-3', while in the mutant construct, the thymidine was replaced by guanine to form an AP-1 mutant, 5'-TGAGGCA-3' termed pT2G (the mutated base is underlined) (Fig. 4). The mutated promoter/luciferase construct, pT2G, contains a single site mutation in the 1.3-kilobase pair rat promoter region. Transfection assays in the various cell types with the wild-type vector show induction of luciferase activity by TGFβ; however, the mutated AP-1 promoter/reporter construct fails to show TGFβ-induced transcriptional activation (Fig. 4).

Transcription Factor Decoy Experiments in CCL64 Cells—We next wished to investigate whether the AP-1 site of the promoter region is specifically responsible for TGFβ-mediated induction of endogenous clusterin expression in CCL64 cells. To address this question, we designed a 23-mer oligonucleotide containing either the wild-type (AP-1) or mutated (T2G) AP-1-binding site (Fig. 5C). These 23-mer oligonucleotides were transfected into CCL64 cells and used as transcriptional factor decoys in experiments analyzing TGFβ-mediated regulation of clusterin mRNA levels (Fig. 5A) and promoter transactivation (Fig. 5B). As shown in Fig. 5A, introduction of the wild-type AP-1 oligonucleotide into CCL64 cells dramatically reduced clusterin mRNA level upon TGFβ stimulation, while the T2G mutated AP-1 oligonucleotide had no effect. In addition, when the AP-1 or T2G oligonucleotides are transiently co-transfected in excess (100:1 nmol) with the full-length pRAL reporter construct (Fig. 5B), only the wild-type AP-1 inhibits TGFβ-induced luciferase activity. These experiments indicate that TGFβ-mediated induction of endogenous clusterin also involves the AP-1-binding site.

Protein Kinase C Is Involved in Mediating TGFβ-induced Clusterin Gene Expression—Previous research has suggested that several protein kinases, including protein kinase C (PKC), may participate in mediating TGFβ signaling although their functions and substrates remain to be elucidated (17). We were interested in investigating if the PKC signaling pathway might be involved in mediating TGFβ-mediated regulation of clusterin gene expression. To address this question, we used two specific pharmacological inhibitors of PKC, namely GF109203X and calphostin C (48, 49), to block TGFβ-mediated regulation of clusterin gene expression. As shown in Fig. 6A, pretreatment of CCL64 cells with a specific PKC inhibitor, GF109203X (48), blocked the ability of TGFβ to induce clusterin gene expression in a dose-dependent manner. Another
and in serum-starved G0-arrested cells. This suggests that this occurs in both rapidly growing, asynchronous cells
yses (Fig. 6) and promoter driven luciferase activity was normalized to β-galactosidase activity of the SV40 β-galactosidase plasmid, and the induction fold was calculated as described in the legend to Fig. 3. C, sequences of the 23-mer oligonucleotides containing either an AP-1-binding site (AP-1) or a T to G mutated AP-1 site (T2G). The position of the oligonucleotide in the promoter region is also shown in the figure. The bold faced sequence represents the consensus AP-1-binding site and the underlined indicates the base that was mutated.

specific PKC inhibitor, calphostin C (49), at concentrations of 50 and 100 nM, was also effective in inhibiting TGFβ-induced clusterin gene expression as determined by Northern blot analyses (Fig. 6B) and promoter driven luciferase activity in CCL64 cells (Fig. 6C). Calphostin C at 100 nM was also effective in inhibiting TGFβ-induced promoter activity in HeLa, 3TP, 10T1/2, and BAEC cells (data not shown). Down-regulation of PKC activity by treatment of the cells with 200 ng/ml phorbol myristate acetate (PMA) for 20 h inhibited TGFβ-induced clusterin mRNA synthesis (data not shown). However, stimulation of PKC in CCL64 cells with 10–100 ng/ml PMA for 0.5–10 h had no effect on clusterin mRNA levels with or without TGFβ stimulation (data not shown).

DISCUSSION

Clusterin is a multifunctional protein that has been implicated in homeostatic control of lipoprotein metabolism, tissue repair and remodeling, sperm maturation, inhibition of complement mediated cell lysis, and epithelial cell differentiation (30). Expression of clusterin is well regulated during development, cell differentiation, and tissue remodeling, and can be modulated by cytokines, such as TGFβ (31, 50, 51). Our previous studies have shown that TGFβ enhances clusterin protein synthesis and RNA levels, and induces the translocation of a cytosolic form of clusterin to the nucleus in CCL64 cells (11). These effects of TGFβ on clusterin led us to investigate the mechanisms of regulation of clusterin gene expression by TGFβ. Our data demonstrate that TGFβ rapidly induces clusterin mRNA levels in a variety of cell lines, including epithelial (CCL64 and HeLa), fibroblast (10T1/2 and 3TP), and BAEC. The induction of clusterin by TGFβ in mink lung epithelial cells (CCL64) occurs in both rapidly growing, asynchronous cells and in serum-starved G0-arrested cells. This suggests that this induction is not cell cycle dependent since it occurs not only during quiescence but also throughout the cycle. These results also suggest that the induction of clusterin is not secondary to TGFβ-induced growth arrest because TGFβ is capable of inducing clusterin in an already G0-arrested cell population, as well in 3TP cells which are not growth arrested by TGFβ (44). Clusterin mRNA can be induced as early as 30 min after TGFβ stimulation and remains elevated for at least 24 h in the presence of TGFβ. This induced expression pattern of clusterin mRNA is similar to that observed with other stimuli where mRNA levels peak within several hours and remain elevated for over 24 h following stimulation (31, 50).

There is over 80% similarity within the first 150 base pairs of the 5′-upstream regulatory region between the human and rat clusterin genes (40). Two conventional cis-elements, Sp1 and AP-1, have been found in the regulatory region of clusterin genes from human, rat, quail, and mouse (39, 40). Several TGFβ-inhibitory elements have also been identified in the first intron of the rat clusterin gene and in the upstream regulatory region of the avian clusterin gene (38, 40, 41). These cis-elements have been implicated in the regulation of clusterin gene expression by various stimuli (37–41). For example, the T64 gene of quail embryo fibroblasts, corresponding to clusterin in mammals, contains an AP-1-binding site located at position −25 to −19 of the single transcriptional start site. Promoter transactivation of the T64 gene has been shown to be significantly induced by an active v-src oncoprotein, and the v-src response requires the AP-1 site and a purine-rich element (39). There is, however, no evidence to indicate that these cis-elements in the promoter region of clusterin genes regulate TGFβ induction.

In the present study, a 1.3-kilobase rat clusterin promoter region has been used for identifying cis-elements mediating TGFβ signaling. This promoter contains AP-1, AP-2, and Sp1 consensus binding sites. Our deletional analyses demonstrate that the AP-2 and Sp1 consensus sites, located at position −124
and −372 from the transcriptional start site, respectively, are not involved in promoter transactivation by TGFβ. However, removal of the AP-1 consensus site, located at position −73 to −79 relative to the transcription start site, abates TGFβ induction of promoter transactivation. The data indicate that TGFβ modulates clusterin gene expression via an AP-1-binding site, and that this AP-1 site is required and sufficient for promoter transactivation by TGFβ. The importance of the AP-1 site in the induction of the clusterin gene by TGFβ is confirmed by our mutagenesis experiments of the AP-1 site in the clusterin promoter region. A single base pair mutation, T to G in the AP-1-binding sites in the collagenase gene has also been shown to be regulated by TGFβ in a cell type specific manner through an AP-1 site.

In the present study we demonstrate that inhibition of PKC activity by two distinct types of PKC inhibitors blocked TGFβ-induced clusterin mRNA levels and promoter transactivation, thus providing further support for the role of AP-1 in TGFβ regulation of clusterin gene expression. Stimulation of PKC activity by treatment of the cells with PMA, however, had no effect on clusterin mRNA levels or TGFβ-induced clusterin mRNA levels. These results suggest that activation of PKC alone is not sufficient to coordinate phosphorylation of nuclear proteins in CCL64 cells, whereas TGFβ stimulates nuclear protein phosphorylation (20). These results are also similar to those observed in activated T cells where full activation of AP-1 requires both the calcium- and PKC-dependent pathways (50). Perhaps TGFβ-inducible clusterin gene expression requires multiple signaling pathways to fully activate...
AP-1 and that inhibiting one of these signaling cascades (i.e., PKC) results in insufficient activation of AP-1 to induce proper transcriptional regulation.

Recent studies in TGFβ signaling indicate that binding of TGFβ to its dimeric receptor complex, composed of the type I and type II receptors, initiates a serine/threonine phosphorylation cascade that involves the Smad proteins. These signaling molecules are phosphorylated on serine and threonine residues by the type I TGFβ receptor and once activated translocate to the nucleus where they are postulated to interact with other Smad proteins and/or transcription factors to initiate target gene expression (65). Future research will be directed at determining how these Smad proteins interact with members of the AP-1 family to regulate gene transcription and how PKC regulates this interaction.

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