Response Gene to Complement 32, a Novel Regulator for Transforming Growth Factor-β-induced Smooth Muscle Differentiation of Neural Crest Cells*†‡§

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We previously developed a robust in vitro model system for vascular smooth muscle cell (VSMC) differentiation from neural crest cell line Monc-1 upon transforming growth factor-β (TGF-β) induction. Further studies demonstrated that both Smad and RhoA signaling are critical for TGF-β-induced VSMC development. To identify downstream targets, we performed Affymetrix cDNA array analysis of Monc-1 cells and identified a gene named response gene to complement 32 (RGC-32) to be important for the VSMC differentiation. RGC-32 expression was increased 5-fold after 2 h and 50-fold after 24 h of TGF-β induction. Knockdown of RGC-32 expression in Monc-1 cells by small interfering RNA significantly inhibited the expression of multiple smooth muscle marker genes, including SM α-actin (α-SMA), SM22α, and calponin. Of importance, the inhibition of RGC-32 expression correlated with the reduction of α-SMA while not inhibiting smooth muscle-unrelated c-fos gene expression, suggesting that RGC-32 is an important protein factor for VSMC differentiation from neural crest cells. Moreover, RGC-32 overexpression significantly enhanced TGF-β-induced α-SMA, SM22α, and SM myosin heavy chain promoter activities in both Monc-1 and C3H10T1/2 cells. The induction of VSMC gene promoters by RGC-32 appears to be CArG-dependent. These data suggest that RGC-32 controls VSMC differentiation by regulating marker gene transcription in a CArG-dependent manner. Further studies revealed that both Smad and RhoA signaling are important for RGC-32 activation.

Alterations in the differentiated state of the VSMC* are known to contribute to a number of major cardiovascular diseases in humans including atherosclerosis, systemic and pulmonary hypertension, and restenosis (1–7). In many of these diseases, it is the ability of VSMCs to modulate their phenotype that leads to pathology. Unlike skeletal or cardiac muscle, VSMCs exhibit a wide variation of phenotypes both in vitro and in vivo (8). The ability of VSMCs to change from a contractile to a synthetic (proliferative) phenotype, while entering the cell cycle and proliferating, may underlie some of the pathological findings in the vascular proliferative disorders mentioned above (9). These dedifferentiated cells display a phenotype closely resembling that of developing VSMCs observed during embryonic angiogenesis (10). Therefore, understanding the molecular mechanisms that control VSMC development and differentiation will provide fundamental insights into several pathological processes, including atherosclerosis, and eventually will lead to the identification of targets for therapeutic intervention.

VSMCs are derived from at least three precursor cell types, depending on the timing and location of formation (11, 12). The three major sources of VSMCs outside of the cardiac circulation in the vertebrate embryo are the neural crest, mesodermal precursors, and endothelium or endothelial stem cells. The other sources of vascular SMC include proepicardial organ and the dermamyotome of the somite (13, 14). Neural crest stem cells are the primary precursor cells that form VSMCs in parts of the cranial circulation, cardiac outflow tracts, and great vessels (15, 16). Mouse mutants defective in neural crest formation exhibit severe defects in cardiac development reminiscent of DiGeorge syndrome (17), underscoring the importance in understanding how VSMCs develop from these precursors. Several factors have been shown to serve as intrinsic and extrinsic fate determinants for neural crest cells (18–25), but the molecular mechanisms controlling their migration and differentiation remain largely unknown. The in vivo signals that specify neural crest commitment to the VSMC lineage have not been fully identified, but TGF-β plays an important role both in vivo and in vitro (26–28).

TGF-β family members transmit signals through transmembrane serine-threonine kinase receptors (29). The TGF-β type II receptor (TβRII) binds ligand and recruits and phosphorylates the type I receptor, activating its kinase activity. Activated type I receptor then binds and phosphorylates downstream signaling intermediates called Smads. Activated Smads translocate to the nucleus where they regulate target gene transcription (30).

Recent studies have shown that specific inactivation of TGF-β signaling in neural crest stem cells results in cardiovascular SMC defects. The TβRII knock-out in neural crest cells

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The abbreviations used are: SMC, smooth muscle cell; VSMC, vascular SMC; TGF-β, transforming growth factor-β; TβRII, TGF-β type II receptor; RGC-32, response gene to complement 32; siRNA, small interfering RNA; α-SMA, SM α-actin; MHC, myosin heavy chain; SRF, serum-response factor; RT, reverse transcription; qPCR, quantitative PCR.

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abolished the development of smooth musculature at embryonic day 10.5. At later developmental stages, neural crest cells that failed to adopt a smooth muscle cell fate underwent apoptotic cell death. These results suggest that the smooth muscle α-actin-positive cells generated from TGF-β-treated neural crest cells in culture indeed represent SMCs (31). A later report from a different group using the same strategy to mutate TβRII in neural crest failed to identify SMC defects, possibly due to an in vivo compensatory mechanism or the use of a different TβRII-floxed mouse line, as discussed by the authors (32). The function of TGF-β signaling on VSMC differentiation from neural crest cells remains controversial. Our in vitro studies have shown that TGF-β robustly induced expression of VSMC markers in neural crest Monc-1 cells (33). Further studies showed that Smad and RhoA play critical roles in mediating TGF-β-induced VSMC differentiation (34). Downstream targets of TGF-β signaling important for VSMC differentiation from neural crest cells, however, remain to be determined.

In the present study, by performing Affymetrix cDNA array analysis and a small RNA interference (siRNA) assay, we have identified a protein, namely response gene to complement (RGC)-32, to be critical for TGF-β-induced VSMC differentiation. Further studies showed that RGC-32 controls VSMC differentiation by regulating the transcription of SMC-specific genes.

EXPERIMENTAL PROCEDURES

Cell Culture—Monc-1 cells were grown as described (35). C3H10T1/2 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, glucose, and l-glutamine supplemented with penicillin.

Mouse RGC-32 Expression Vector Construction—Mouse RGC-32 cDNA was amplified from mRNA extracted from TGF-β-treated Monc-1 cells. The 5’ primer included a BamHI restriction site for cloning, a Kozak sequence, and a T7 tag followed by a RGC-32 cDNA sequence. The 3’ primer included RGC-32 cDNA sequence, a stop codon, and an XbaI restriction site. RGC-32 full-length cDNA was amplified with Vent DNA polymerase (New England Biolabs). For cloning, both pcDNA 3.0 vector and amplified RGC-32 cDNA were digested with BamHI and XbaI and then purified, followed by ligation using T4 DNA ligase (New England Biolabs). The cloned cDNA was verified by sequencing. RGC-32 overexpression in Monc-1 cells was confirmed by Western blot using T7 antibody (Novagen).

RNA Interference—To knock down RGC-32 expression, Monc-1 cells were transfected with RGC-32 siRNA using Lipofectamine 2000. 6 h later, the cells were treated with TGF-β (5 ng/ml) for 24 h. Cells were then collected for analysis of RGC-32 and SMC marker expression. RGC-32 siRNAs (5’-CCUGCCCAUUCUUGGUUCACAUAA-3’) were purchased from Invitrogen.

Reverse Transcription-PCR (RT-PCR) and Quantitative PCR (qPCR)—RT-PCR and qPCR were performed as described previously. RGC-32 primer sequences were: 5’-AAGCCCGCCT-CAGGCAGAAGCAG-3’ (forward) and 5’-CATACTTGTCT-AAGTCCGT-3’ (reverse). Primers for SMC marker genes α-SMA, SM22α, calponin, and SMC-nonspecific gene c-fos were previously described (33).

Transfection and Luciferase Reporter Assay—α-SMA wild type and CαG box mutants and myosin heavy chain (SM-MHC) promoter luciferase reporter constructs were generously provided by Dr. Gary Owens (36). SM22α promoter luciferase reporter construct was previously described (37). pcDNA or RGC-32 expression vector co-transfection with promoter construct into Monc-1 or C3H10T1/2 cells followed by luciferase assay was performed similarly as described previously (33), except that the cells were growth factor- (Monc-1 cells) or serum- (10T1/2 cells) starved and then TGF-β-treated for 48 h before luciferase assay.

Statistical Analysis—All values are expressed as mean ± S.E. Data were analyzed using analysis of variance with pairwise comparisons between groups. A level of p values < 0.05 was considered statistically significant.

RESULTS

We have previously described that Smad and RhoA are the two pathways critical for TGF-β-induced SMC differentiation from Monc-1 cells. To identify the earliest downstream targets that may initiate the differentiation program, we treated Monc-1 cells with TGF-β for 24 h and then performed microarray analyses using Affymetrix chips. RGC-32 was identified to be highly up-regulated (15-fold) by TGF-β (data not shown). RGC-32 was first identified in oligodendrocytes in response to complement activation (38). It is a 14-kDa protein expressed in many adult human tissues including heart, brain, liver, skeletal muscle, placenta, kidney, and pancreas (39), and it is overexpressed in colon cancer and many tumors (40). Functionally, RGC-32 has been shown to play a role in cell cycle activation. It is a substrate and regulator of cyclin-dependent kinase p34Cdc2 (39, 41). Whether RGC-32 is involved in VSMC differentiation, however, remains to be determined.

To confirm the TGF-β induction of RGC-32, we performed regular RT-PCR and qPCR to determine RGC-32 expression in Monc-1 cells. We found that TGF-β significantly up-regulated RGC-32 expression in Monc-1 cells. qPCR showed that RGC-32 was significantly up-regulated as early as within 2 h of TGF-β induction. The induction was increased 50-fold after 24 h of treatment and remained increased for up to 4 days, the longest time tested (supplemental Fig. S1).

To determine whether RGC-32 is functionally important for SMC differentiation, we used siRNA to knock down RGC-32 expression in Monc-1 cells and then treated the cells with TGF-β. As shown in Fig. 1, RGC-32 siRNA effectively inhibited RGC-32 expression, and at the same time, blocked the expression of marker genes including α-SMA, SM22α, and calponin (Fig. 1A). Importantly, inhibition of RGC-32 expression correlated with the reduction of α-SMA but did not affect SMC-nonspecific c-fos gene expression (Fig. 1B). Control siRNA did not inhibit the expression of any genes. These data suggest that RGC-32 is essential for TGF-β-induced SMC differentiation from Monc-1 cells.

Because transcriptional regulation is an important process in SMC differentiation, we decided to test whether RGC-32 regulates SMC marker promoter activity. We cloned RGC-32 cDNA into pcDNA expression vector and then co-transfected the expression vector with α-SMA, SM22α, or SM-MHC promoter
constructs into Monc-1 cells. Luciferase assays showed that RGC-32 significantly enhanced TGF-β-induced promoter activities of all the three marker genes (Fig. 2, A–C).

To determine whether the effect of RGC-32 is an isolated phenomenon in Monc-1 cells or functions in other systems as well, we performed similar experiments in another cell line, C3H10T1/2 fibroblasts (10T1/2). 10T1/2 cells have been used as a model for SMC differentiation by many groups (37, 42–45). RGC-32 was significantly up-regulated with TGF-β induction (supplemental Fig. S2). Functional analysis revealed that RGC-32 regulated α-SMA and SM22a in 10T1/2 cells in a manner similar to Monc-1 cells (Fig. 2, D and E). Interestingly, RGC-32 appeared to be more potent in regulating SM-MHC promoter activity in 10T1/2 than in Monc-1 cells (compare Fig. 2F with 2C), suggesting that the mechanism for SMC differentiation of Monc-1 cells is different from that of 10T1/2 cells.

SRF and its cofactor myocardin play important roles in SMC differentiation (36, 46–55). TGF-β has been shown to increase SRF binding to SM22α and SM γ-actin promoter in 10T1/2 cells (56, 57). To determine whether RGC-32 function is associated with SRF, we co-transfected RGC-32 expression vector with wild type α-SMA promoter (from −2.6 to +2.8 kb) construct or the construct with mutations at CArG box(es) in the promoter region (CArGm(A+B)) or in the first intron (CArGmIntr) was co-transfected with pcDNA or RGC-32 expression vector into Monc-1 cells as indicated. Luciferase assay was performed. Data shown were the representative results of three independent experiments. *, p < 0.01 for comparison with wild type α-SMA promoter activity in the presence of RGC-32.

and RhoA signaling are important for RGC-32 activation, we used Smad7 to block Smad signaling, and C3 exotoxin to block RhoA signaling. We found that both Smad7 and C3 exotoxin significantly inhibited TGF-β-induced RGC-32 expression in Monc-1 cells (Fig. 4), suggesting that both Smad signaling and RhoA are important for RGC-32 activation during TGF-β-induced SMC differentiation from Monc-1 cells.

DISCUSSION

Our previous studies have established an in vitro SMC differentiation model by using TGF-β-treated neural crest stem cell Monc-1 (33, 34). In this report, by performing Affymetrix microarray analyses, we identified RGC-32 to be essential for TGF-β-induced SMC differentiation. Blockade of RGC-32 expression using siRNA significantly inhibited the expression...
of multiple SMC-specific genes but did not inhibit SMC-nonspecific gene. Further studies demonstrated that RGC-32 controls SMC differentiation by regulating SMC marker gene transcription. Overexpression of RGC-32 in both Monc-1 and 10T1/2 cells significantly up-regulated TGF-β-induced α-SMA, SM22α, and SM-MHC promoter activities.

Expression of most SMC marker genes has been shown to be regulated by multiple CArG elements and SRF (49, 51, 53–55). RGC-32 regulation of α-SMA promoter is also CArG-dependent, suggesting that RGC-32 may participate in SRF-associated regulatory network for SMC differentiation. Whether RGC-32 is a cofactor of SRF in neural crest cells is a subject of future study.

TGF-β-induced SMC differentiation from Monc-1 cells requires both Smad and RhoA signaling (33, 34). Current studies demonstrate that both Smad and RhoA are important for RGC-32 activation. Therefore, we conclude that TGF-β-induced SMC differentiation from Monc-1 cells is regulated as follows. TGF-β first activates Smad and RhoA signaling, which further activates RGC-32. RGC-32, together with other transcriptional factors including SRF and Smads, then turns on SMC maker gene transcription and initiates SMC differentiation.

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