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REVIEW

Transforming growth factor-β and smooth muscle differentiation

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Abstract

Transforming growth factor (TGF)-β family members are multifunctional cytokines regulating diverse cellular functions such as growth, adhesion, migration, apoptosis, and differentiation. TGF-βs elicit their effects *via* specific type Ⅰ and type Ⅱ serine/threonine kinase receptors and intracellular Smad transcription factors. Knockout mouse models for the different components of the TGF-β signaling pathway have revealed their critical roles in smooth muscle cell (SMC) differentiation. Genetic studies in humans have linked mutations in these signaling components to specific cardiovascular disorders such as aorta aneurysm and congenital heart diseases due to SMC defects. In this review, the current understanding of TGF-β function in SMC differentiation is highlighted, and the role of TGF-β signaling in SMC-related diseases is discussed.

Key words: Transforming growth factor β; Smad; Smooth muscle cell; Differentiation; Cardiovascular diseases

TGF-β SIGNALING TRANSDUCTION

Transforming growth factor-β (TGF-β) is the founding member of the TGF-β superfamily that comprise TGF-βs, activins, bone morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs)[1]. Three TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) have been identified in mammals. In most cases, these isoforms exhibit similar functional properties and regulate various cellular activities including cell growth, differentiation, apoptosis and extracellular matrix synthesis in endothelial cells and vascular smooth muscle cells (SMCs)[2-8].

TGF-β ligands are synthesized as latent precursor molecules (LTGF-β), which are activated *via* proteolytic cleavage by endoproteases such as furin[9]. Active TGF-β signaling is transmitted through two types of transmembrane serine/threonine protein kinase receptors: TGF-β type Ⅰ (TβR Ⅰ) and type Ⅱ (TβR Ⅱ)[1,10-12]. TGF-β first binds to TβR Ⅱ with the assistance of the membrane-anchored proteoglycan betaglycan TGF-β receptor Ⅲ (TβR Ⅲ)[13], which leads to heterotetrameric complex formation with TβRI, resulting in TβRI phosphorylation[14,15]. TβRI (also known as activin receptor-like kinase

5; ALK5) transduces TGF-β signaling in most cell types although the signaling can also be mediated by ALK1 or other type I receptors in certain cell types[16,17]. Activated TβRI propagates signaling by recruiting and phosphorylating receptor-regulated Smad (R-Smad) proteins. ALK5 phosphorylates Smad2 and Smad3, while ALK1 phosphorylates Smad1, Smad5, and Smad8. Activated Smads form a complex with the common Smad (Smad4) and then are translocated into the nucleus, where they regulate target gene expression by binding to regulatory promoter DNA alone or interacting with other transcription factors[18,19].

Smad3 homomer can form DNA-binding complexes through its MH1 domain independent of Smad4. But Smad2 cannot bind to DNA without Smad4 because of the lack of the additional 30 amino acids present in Smad3 MH1 domain. Smad4 and phosphorylated Smad3 bind multiple 5'-AGAC-3' sequences called Smad binding elements (SBEs) and GC-rich sequences[20]. Smad2 and Smad3 interact with a number of common and distinct transcription factors for SBE selectivity and specific gene transcription[21]. In most cases, Smad-binding transcription factors can function independent of Smads in controlling a specific gene transcription. However, Smad interacts with these transcription factors to modulate their transcriptional activity by recruiting co-activators or co-repressors[20,22,23]. For example, Smads recruit transcription coactivator p300/CBP, which has histone acetyltransferase activity, to facilitate the initiation of transcription[20]. In addition to p300/CBP, various other transcription factors such as Forkhead, homeobox, zinc-finger, AP1, Ets, and basic helix-loop-helix families have also been shown to act in concert with Smad proteins[24,25]. The diversity of Smad/co-factor combinations enables the regulation of the transcription of a vast amount of target genes. The differential expression of these factors in different cells are thought to contribute, at least in part, to the cell type-specific responses observed upon TGF-β stimulation[19].

TGF-β/Smad signaling pathway is regulated in multiple steps by different factors. SARA (SMAD anchor for receptor activation) presents R-Smads to the activated receptor complexes[26], while TMEPAI (transmembrane prostate androgen-induced protein) sequesters R-Smad proteins from active participation in TGF-β signaling[27]. Inhibitory Smad (I-Smad), Smad6 or Smad7, inhibits R-Smad binding to TGF-β receptor[28-30]. Smad Phosphorylation is reversed by phosphatases such as PPM1A and PDP in order to create a rapid activation-deactivation cycle[31-33]. Moreover, activated Smad proteins may be ubiquitinized by E3 ligases for proteasomal degradation[34,35]. In addition, transcriptional repressors Ski and SnoN also regulate TGF-β signaling by interacting with Smad proteins[36,37].

In addition to the canonical Smad signaling pathway that directly regulates the transcription of Smad-dependent target genes, TGF-β function can also be mediated by Smad-independent pathways including MAPK signaling pathways, such as p38 MAPK and c-Jun NH2-terminal kinase, phosphatidylinositol 3-kinase/Akt pathway, and Wnt signaling[38].

TGF-β SIGNALING IN SMOOTH MUSCLE DIFFERENTIATION DURING EMBRYONIC DEVELOPMENT

SMC differentiation is an integral part of embryonic vascular development. Vascular development in the embryo starts with the formation of a primitive vascular network from endothelial precursors through a process known as vasculogenesis. This primary vessel network undergoes angiogenesis to grow into a complex vascular system through branching and remodeling[39]. Recruitment and differentiation of SMC progenitor cells are essential process for both vasculogenesis and angiogenesis. The function of SMCs is to stabilize nascent vessels by inhibiting excessive endothelial cell proliferation and migration. In addition, SMCs express vasoactive peptides, growth factors and cytokines which are important for the overall function of vasculature. After birth, the principal function of SMCs is to regulate pulse pressure and blood flow through contraction[40]. SMCs are capable of reversibly modulating their phenotype during postnatal development and can de-differentiate into proliferative, matrix synthetic cells in response to vascular injury[41,42]. Abnormal SMC differentiation or function contributes to a number of cardiovascular disorders including congenital heart diseases, aortic aneurysm, atherosclerosis, hypertension, and restenosis[42-51].

TGF-β signaling plays pivotal roles in SMC differentiation during vascular development as well as phenotypic switching in disease states[52]. The importance of TGF-β signaling pathway in SMC differentiation during embryonic development has been demonstrated by numerous studies[53]. Gene-targeting studies in mice have shown that a loss of TGF-β signaling components generally leads to abnormal differentiation and maturation of the primitive vascular network, resulting in defective vessels losing integrity of the vessel wall. One of the defects is the failure of smooth muscle cell recruitment and/or differentiation[54]. 50% of mice with both alleles of TGF-β gene deleted die in utero around 10.5 dpc due to abnormalities in yolk sac vessel development. The vascular defects are caused, at least in part, by the failed differentiation of mesenchymal precursors into vascular SMCs[55]. In young mice with one allele of the TGF-β gene deleted, the levels of both TGF-β and smooth muscle differentiation markers are reduced as compared with that of wild-type mice. This regulation of smooth muscle differentiation by TGF-β also occurs dynamically in the adult animals[56]. Quantitative immunofluorescence data in rat arteries demonstrate that levels of smooth muscle differentiation markers correlate with the levels of TGF-β expression[57].

TβR Ⅱ is unique and essential for TGF-β signaling[1]. SMC-specific deletion of TβR Ⅱ gene is the best method to generate mice with ablation of TGF-β signal-

ing in SMCs. Langlois and colleagues have generated mice with conditional deletion of the gene in cells expressing SMC-specific marker SM22α. Their results have shown that all SM22α-Cre/TβR Ⅱ-floxed embryos die between E14.5 and the end of pregnancy. All mutant embryos display profound vascular abnormalities in the descending thoracic aorta including irregular thickness, occasional aneurysms and elastic fiber disarray. Importantly, VSMC differentiation is impaired in the descending thoracic aorta in these embryos. TβR Ⅱ gene deletion in the VSMCs of the descending thoracic aorta diminishes the number of smooth muscle α-actin (α-SMA)-positive VSMCs in the media at E11.5. These results suggest that TGF-β plays an irreplaceable role in the differentiation of VSMCs in the descending thoracic aorta during mouse development[58].

The role of TβR Ⅱ in SMC differentiation has also been demonstrated by tissue-specific knockout of TβR Ⅱ gene in neural crest cells. During embryonic development, neural crest cells migrate to various locations within the embryo and differentiate into non-neural tissues. One subpopulation named cardiac neural crest can differentiate to SMCs of ascending aorta and great arteries by a number of growth factors including TGF-β[59,60]. TGF-β function in this process is demonstrated by neural crest-specific ablation of TβR Ⅱ using Cre-loxP system. TβR Ⅱ protein is specifically deleted in neural crest and neural crest-derived cells by mating TβR Ⅱ-floxed mice with Wnt1-Cre mice. Mouse hearts with TβR Ⅱ deletion display truncus arteriosus together with ventricular septal defects. In addition, the mutant mice exhibit abnormal patterning of the arteries arising from the aortic arch, the main cause of mortality in human DiGeorge patients. Importantly, although the mutant neural crest cells are able to migrate and form aorto-pulmonary septum at E10.5, they do not contribute to the development of the smooth musculature and fail to adopt a smooth muscle cell fate[61]. The absence of neural crest-derived smooth muscle cells in mutants explains the defective separation of the aorta from the pulmonary trunk, leading inevitably to a truncus arteriosus. Although a later report using the same strategy has failed to identify SMC defect, which is likely due to, as discussed by the authors, an in vivo compensatory mechanism or the use of a different TβR Ⅱ-floxed mouse line[62], TGF-β signaling appears to be crucial in SMC differentiation from neural crest cell during embryonic development.

ALK5, a type I receptor, has been shown to be involved in the induction of epicardial to mesenchymal cells, one of the processes by which differentiated smooth muscle cells are produced. Ablation of Alk5 in epicardial lineages using Gata5-Cre mouse lines results in the failure of TGF-β-induced epicardial to mesenchymal cell transition. Late-term mutant embryos lacking epicardial Alk5 display defective formation of the SMC layer around coronary arteries and aberrant formation of capillary vessels in the myocardium[63]. In addition to ALK5, ALK1 is also required for the differentiation and recruitment of vascular SMCs to the vascular endothelium cells because ALK1 knockout embryos contain no VSMCs[64-68]. Mice mutant for ALK1 develops arterio-venous malformations (AVMs), a serious condition characterized by shunting between the arterial and venous circulations.

Endoglin (also known as CD105) is a homodimeric membrane glycoprotein located on cell surface of vascular endothelial cells, hematopoietic cells, neural crest stem cells, *etc*[69-71]. Endoglin has been identified as a part of the TGF-β receptor complexes and can be co-precipitated with TβR Ⅱ and TβRI in endothelial and leukemic cells[72-74]. Endoglin has a pivotal function in the development of the cardiovascular system and in vascular remodeling. Mice lacking endoglin gene die during embryonic development due to cardiovascular abnormalities[68,75]. In contrast to the mice lacking TGF-β or its signaling receptors, the process of vasculogenesis occurs normally in endoglin mutant embryos. However, the second stage of vascular development, angiogenesis, is affected as shown by the absence of organized vessels in yolk sacs. Therefore, endoglin is important in angiogenesis rather than in vasculogenesis. Importantly, disrupted development of SMC in the yolk sac is observed in endoglin null mice. One important mechanism underlying the limited number of SMC in the vessel walls is the reduced availability of TGF-β protein levels. In endoglin knockout mice, the lacking of TGF-β pathway in endothelial cells of the yolk sac leads to the decreased phosphorylation of Smad2 in the mesothelial layer, which eventually inhibits the recruitment and differentiation of mesenchymal cells into VSMCs[76].

Smad proteins are important components of TGF-β signaling pathway[1,4,22,23]. Smad5 is expressed predominantly in mesenchyme and somites during embryogenesis and in many tissues of the adult. The Smad5 homozygous mutant embryos (Smad5ex6/ex6) exhibit phenotypes similar to those of TGF-β and TβR Ⅱ knockouts. Smad5ex6/ex6 embryos die at E10.5-11.5 due to defects in angiogenesis which requires extensive interactions of endothelial cells with pericytes or smooth muscle cells. Smad5ex6/ex6 embryos have dilated blood vessels, and the layer of endothelial cells is dissociated from mesenchymal cells, suggesting that the interaction between the endothelial and mesenchymal cells is affected. Many Smad5ex6/ex6 embryos suffer massive apoptosis of mesenchymal cells. The abnormal blood vessels display a decrease in the thickness of SMC layer, indicating that the differentiation of mesenchymal cells into SMC is impaired in Smad5ex6/ex6 mutants[65].

MOLECULAR MECHANISMS OF TGF-β-INDUCED SMC DIFFERENTIATION

SMCs are defined by specific molecular markers and contractile functions. Smooth muscle α-actin (α-SMA) and SM22α are early markers of developing SMCs while calponin, caldesmon, and smooth muscle myosin heavy chain (SMMHC) are late markers. The principal function of SMCs is to regulate pulse pressure and blood flow through contraction[40]. In order to understand the under-

lying mechanisms of TGF-β-induced SMC differentiation, several *in vitro* models have been developed including primary cultured VSMCs[77], C3H10T1/2 (10T1/2, a multipotent mouse embryonic mesenchymal cell line)[78], and neural crest Monc-1 cells (pluripotent neural crest stem cells)[79], *etc*. TGF-β has been shown to induce these cells to change into a polarized and elongated SMC morphology accompanied by an up-regulation of SMC contractile proteins[78-84]. These models have significantly contributed to the understanding of transcriptional regulation of genes essential for SMC function. Three TGF-β responsive elements have been identified: the TGF-β control element (TCE), the SBE, and the CArG box. Mutation of any of these elements abolishes TGF-β induction. In addition, crosstalk between TGF-β and Notch signaling is found to be involved in SMC differentiation. Moreover, microRNA is recently emerging as an important regulator for TGF-β-induced SMC differentiation.

TCE/KLF4/KLF5

TCE is a cis-element in SMC promoter region and highly conserved across species in multiple SMC marker genes including α-SMA, SM22α, SMMHC and calponin[85]. Mutation of TCE in α-SMA or SM22α promoter region abolishes TGF-β-induced α-SMA and SM22α promoter activity in cultured SMCs[85-87]. The importance of TCE in the regulation of α-SMA and SM22α promoter activity was further studied in transgenic mice with wild-type and TCE-mutant promoters coupled to a LacZ reporter gene. TCE mutations completely block the promoter activity in directing LacZ transcription in arterial SMCs[86,87]. Both α-SMA and SM22α TCE form a TGF-β-dependent complex with nuclear proteins in electrophoretic mobility shift assays (EMSAs)[86,88]. Mutation of SM22α TCE completely abolishes this complex formation[86]. GKLF/KLF4, a Kruppel-like transcription factor (KLF) containing three C2H2 zinc fingers, specifically binds to SM22α or α-SMA TCE. Interestingly, KLF4 represses rather than activates TCE activity. Overexpression of KLF4 inhibits TGF-β-stimulated increase in SM22α or α-SMA promoter activity in 10T1/2 cells. KLF4-mediated repression of the promoter activity is TCE-dependent because in rat aortic SMCs, KLF4 overexpression inhibits the activity of wild type α-SMA promoter but has no effect on the activity of TCE mutant α-SMA promoter[87]. In addition, inhibition of KLF4 with antisense morpholinos increases α-SMA and SMMHC expression[87]. TGF-β inhibits KLF4 expression in cultured SMCs through induction of microRNA-143 (miR-143) and miR-145, leading to a reduction of KLF4 transcripts and decreased KLF4 protein expression[86,89].

Studies of KLF4 lead to the finding of another Kruppel like factor, a GKLF-related basic transcriptional element-binding protein (KLF5). KLF5 binds specifically to SM22α TCE[86]. Overexpression of KLF5 enhances TGF-β-dependent SM22-LacZ promoter activity in 10T1/2 cells, while reversing KLF4-mediated repression of α-SMA promoter activity induced by SRF in NIH3T3 cells. These studies suggest that TCE may act as an activator or a repressor of SMC marker genes depending on the stoichiometry of specific binding factors.

SBE/Smad signaling

As mentioned earlier, Smads are major intracellular mediators of TGF-β signaling pathway. When Smad2/Smad3 is phosphorylated, they are translocated into nuclear to regulate gene transcription. Smads bind to SBE (CAGA or GTCT) to regulate gene transcription. SBE is an important TGF-β responsive element in the promoter region of SMC marker genes and thus regulates SMC differentiation. Mutation of SBE in SM22α promoter inhibits TGF-β-induced SM22α promoter activity in 10T1/2, Balb3T3 and Monc-1 cells[90,91]. Transgenic embryos with SBE-mutated SM22α promoter show diminished transcription activation potential of the promoter in the arteries[90]. TGF-β induces a nuclear complex bound to the SBE sequence, and mutation of the SBE blocks this inducible interaction, indicating that SBE is required for the formation of the TGF-β-inducible complex. Smad3 and Smad4 but not Smad2 are present in these inducible complexes. The Smad3 binding to the SBE of SM22α promoter in vivo is demonstrated by chromatin immunoprecipitation assay[90]. It appears that Smad3, but not Smad2 or Smad4, activates SBE activity. Smad3 increases the transactivation of SBE reporter but not the mutant SBE reporter. Therefore, Smad3 is the major mediator of TGF-β-induced SM22α transcription, and SBE in the SM22α promoter is a direct target of Smad3.

In addition to Smad, several other pathways such as RhoA also mediate TGF-β signaling. RhoA is a member of Rho GTPase family that has intrinsic GTPase activity and can shuttle between an inactive GDP-bound state and an active GTP-bound state[92]. RhoA is highly expressed in mature VSMCs. RhoA and p160 Rho kinase (ROCK), a downstream effector of RhoA, regulate the expression of α-SMA and SM22α[93]. Overexpression of RhoA or activation of RhoA in cultured VSMCs causes a contractile phenotype and organized arrangement of actin and myosin. On the other hand, inhibition of RhoA leads to a loss of actin and myosin filaments, indicating that RhoA plays a key role in regulating SMC contractile function[94]. RhoA/ROCK regulates the expression and nuclear translocation of SRF in SMCs, and ROCK inhibitor decreases SRF enrichment to CArG regions of α-SMA and SMMHC promoters[95,96]. It appears that RhoA activates SMC marker gene expression *via* both Rock-dependent and independent pathways in rat pulmonary artery SMCs[97]. Our studies show that RhoA regulates TGF-β-induced SMC differentiation *via* modulating Smad signaling. RhoA is activated as early as 5 minutes following TGF-β induction. Inhibition of RhoA blocks TGF-β-induced expression of α-SMA, SM22α and calponin and reverses TGF-β-induced morphology alteration and contractility, indicating that RhoA is essential in TGF-β-stimulated SMC differentiation. Dominant negative RhoA blocks Smad2 and Smad3 phosphorylation,

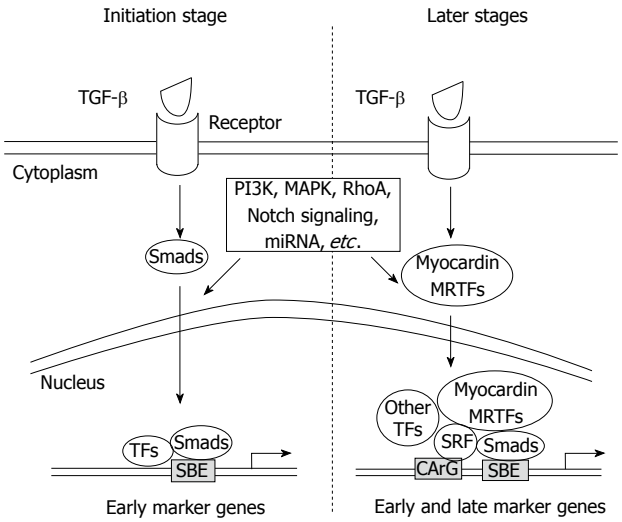

Figure 1 Transforming growth factor-β in smooth muscle cell differentiation. In the initiation stage of smooth muscle cell (SMC) differentiation, transforming growth factor (TGF)-β rapidly activates Smad signaling, leading to the activation of SMC early marker genes and cell fate determination by interacting with other transcription factors (TFs). At later stages, myocardin or MRTFs, *via* interacting with SRF, Smads and other TFs, enforce and accelerate SMC differentiation and maturation. In both the initial or later stages, other signaling pathways including PI3K, MAPK, RhoA, Notch, and miRNA may participate in the regulation of the differentiation process by interacting with TGF-β signaling molecules or downstream targets.

resulting in an impaired nuclear translocation and transcriptional activity, which eventually inhibits SMC marker gene expression. Conversely, constitutively active RhoA significantly enhances Smad-dependent promoter activity[98]. These results suggest that RhoA cross-talks with Smad to regulate TGF-β-induced SMC differentiation.

CArG/SRF and myocardin

Almost all SMC-specific genes have conserved CArG elements with a consensus sequence CC(A/T-rich)$_6$GG in their promoter regions. CArG box, also called serum response element, is involved in TGF-β-induced SMC marker gene expression *via* binding of SRF[42,85,99,100]. Overexpression of SRF increases SMC marker expression in 10T1/2 cells. Moreover, cell morphology changes from flat to elongated shape in SRF-transfected 10T1/2 cells[100]. TGF-β induces SRF protein expression and enhances its binding activity to the CArG boxes in inducing SMC phenotype[85,100,101]. CArG box mutation disrupts SRF binding and completely abolishes TGF-β-induced transcriptional activation of SMC marker genes[100]. SRF appears to interact with Smad3 upon TGF-β induction and regulates Smad3 transactivation of SM22α promoter.

Myocardin is a transcriptional cofactor of SRF and is highly expressed in aortic medial SMCs[102]. Overexpression of myocardin leads to a high induction of calponin and α-SMA with a cell morphological alteration from flat to spindle shape in several cell lines[103-106]. In addition, overexpression of myocardin stimulates SM22α, α-SMA and SMMHC promoter activities in mouse ES cells[104]. siRNA knockdown of myocardin significantly reduces transcriptional activity of α-SMA, SM22α and SMMHC in aortic SMCs[106]. Myocardin-null embryos die at E10.5 and lack the differentiation of vascular SMCs[107]. Myocardin induces SMC marker transcription in a CArG-dependent manner[106]. Moreover, myocardin alone is sufficient to induce a SMC-like contractile phenotype[108].

Myocardin is also involved in TGF-β-induced SMC differentiation. Myocardin appears to directly interact with Smad3 in a CArG box-independent manner. Myocardin enhances TGF-β-induced alteration of cell morphology and SM22α transcription in 10T1/2 cells[90]. Overexpression of myocardin and Smad3, but not Smad2, leads to a synergistic increase of SBE promoter activity. Moreover, myocardin enhances Smad3-mediated activation of SM22α, SMMHC and α-SMA promoter activities[90]. Taken together, both SRF and its transcription cofactor myocardin play important roles in TGF-β-induced SMC differentiation.

Although myocardin is considered to be a master regulator of SMC differentiation, the expression of some SMC-associated genes such as smoothelin-B is independent of myocardin[109,110]. In addition, some progenitor cells such as A404 expressing a low level of myocardin are not converted to SMC phenotype without retinoic acid (RA) induction[106]; Conversely, other SMC progenitors such as 10T1/2 cells can be converted to SMC phenotype by overexpression of myocardin[106], suggesting that a threshold level of myocardin is required for SMC differentiation. In vivo studies show that the expression of early SMC marker genes such as SM22α and α-SMA emerges prior to detectable myocardin mRNA in the embryonic dorsal aorta, indicating that myocardin has a minor role in the initiation of SMC differentiation in some vascular tissues[104,111-113]. A recent report shows that myocardin null embryonic stem cells can readily form vascular SMCs in the setting of chimeric knockout mice. The results from this study provide novel evidence that myocardin is essential for development of visceral SMCs and ventricular myocytes but is dispensable for development of atrial myocytes and vascular SMCs[113]. Our in vitro studies demonstrate that myocardin may not participate in the initiation of TGF-β-induced SMC differentiation because the early SMC markers are induced preceding the induction of myocardin. It appears that Smad3 activation by TGF-β has blocked the expression of myocardin. Smad3 blocks myocardin transcription by interacting with Nkx2.5, which prevents Nkx2.5 from activating myocardin promoter[114]. Our data suggest that Smad may mediate the initiation of TGF-β-induced SMC differentiation, while myocardin is likely to contribute to the maturation of SMCs during a later stage (Figure 1).

TGF-β and notch signaling

Like TGF-β, Notch signaling induces SMC differentiation[115-118]. Once ligands (such as Delta-like or Jagged) bind to Notch receptor (Notch1, Notch2, Notch3 and Notch4), the Notch intracellular domain (NICD) is cleaved and translocated into the nucleus to interact with

the DNA-binding protein CSL (CBF-1, suppressor of hairless, and Lag-1, also known as RBP-Jκ), mastermind-like (MAML), and other transcriptional coactivators to modulate the expression of Notch target genes that regulate cell fate decisions[118]. Numerous data show that Notch induces SMC specific marker expression including α-SMA, SM22α, calponin and SMMHC in a number of cell lines[116,117,119]. Notch signaling specific inactivation in the neural crest causes cardiac outflow tract defects with decreased expression of SMC markers[116]. Although there are four types of Notch receptor, only Notch1 and Notch3 are expressed in VSMCs. In adult Notch3$^{-/-}$ mice, VSMCs show deficiency in postnatal maturation stage. The expression of late stage SMC marker smoothelin B is significantly inhibited in mutant arteries, suggesting a pivotal role of Notch3 in the maturation of VSMCs[120].

It appears that TGF-β cross-talks with Notch signaling in the regulation of SMC differentiation. TGF-β and Notch have cooperative effect on SMC differentiation[121]. In human SMCs, both Jagged1 and Notch induce SMC marker expression. SMCs embedded within collagen matrix exhibit a greater contractile response with both TGF-β and NICD comparing to individual treatment. CBF1 interacts with Smad2/3, which leads to an increased Smad2/3 transcriptional activity. In addition, Notch increases TGF-β-induced binding of Smad2/3 to SMC marker promoter. As most of SMC marker gene promoters contain CBF1 and Smad consensus binding sites, it is possible that NICD/CBF1 complex binding to adjacent promoter region, which provides a cis regulatory signal to promote Smad binding. In addition to the mature SMCs, TGF-β and Notch also show cooperative activity in SMC differentiation of huMSCs and embryonic stem cells. TGF-β induces Jagged1 expression in huMSCs, suggesting that Notch activation mediates TGF-β signaling during huMSC differentiation into SMC. Knockdown of Jagged1 using shRNA inhibits TGF-β-induced SMC marker expression in huMSCs[122]. Although TGF-β and Notch cooperate in most of cases, TGF-β appears to inhibit Notch 3 in SMC differentiation of 10T1/2 cells[123], suggesting that TGF-β and Notch signaling pathways interacts in a cell-specific manner.

MicroRNA

MicroRNA (miRNA) are small non-coding RNAs that function as negative regulators of gene expression by associating with the complementary sequences in the 3' untranslated regions (UTRs) of mRNAs, resulting in mRNA degradation and/or translational inhibition[124,125]. A number of studies have shown that miRNAs plays a role in VSMC phenotype switch[126-130]. TGF-β/BMP regulate around 20 miRNAs[131], which control expression of protein-coding genes associated with epithelial-mesenchymal transition, skeletal muscle cell differentiation, and cell proliferation, *etc*[39,132,133]. miR-143 and miR-145, which are encoded as a gene cluster, target KLF4 and play a critical role in regulating VSMC phenotype[127,134,135]. miR-143 or miR-145 VSMC knock-out mice exhibit abnormal vascular tone and reduced contractile gene expression[134]. The expression of miR-143 and miR-145 is repressed during platelet-derived growth factor (PDGF)-induced VSMC dedifferentiation and during neointimal formation[127]. Recent studies indicate that miR143/145 plays a role in TGF-β-induced SMC differentiation[89,136]. TGF-β stimulates miR143/145 expression in a dose- and time-dependent manner in VSMC. TGF-β-induced miR143/145 expression is myocardin /SRF-, p38-, and Smad4-dependent[136]. Both CArG box and SBE are essential for TGF-β-dependent activation of miR143/145 enhancer[136]. BMP-4 also induces miR143/145 expression. TGF-β and BMP-4 induction of miR143/145 results in down-regulation of KLF4[89]. Interestingly, BMP-4 induces miR143/145 through myocardin-related factor A (MRTFA), but not myocardin, suggesting that TGF-β and BMP4 signaling regulate KLF4 expression through different mechanisms[89].

TGF-β IN SMC-RELATED DISEASES

The principal postnatal function of SMCs is to regulate pulse pressure and blood flow through contraction[40]. SMCs are capable of reversibly modulating their phenotype during postnatal development and can de-differentiate into proliferative, matrix synthetic cells in response to vascular injury[41,42]. TGF-β regulates both SMC differentiation during embryonic development and postnatal phenotypic switching[74,75,137]. Overexpression of TGF-β increases the neointimal formation and smooth muscle proliferation and differentiation in balloon injury models[138,139]. Therefore, it is conceivable that TGF-β plays an important role in the re-differentiation phenomena[140]. TGF-β has been shown to be involved in the development of many cardiovascular diseases including atherosclerosis, congenital heart diseases, aortic aneurysm, hypertension and hereditary hemorrhagic telangiectasia, *etc*[141-143]. Many of these diseases are due to the failed regulation of SMC function or differentiation.

Atherosclerosis

Atherosclerosis is triggered in response to chronic injury to the vascular endothelium by various risk factors. It is a progressive disease characterized by the formation of a plaque in the inner lining of large arteries. VSMC proliferation, migration, and hypertrophy are involved in the development of atherosclerosis. VSMCs play a maladaptive role in the lesion development and the progression of the disease[144]. TGF-β directs the response of SMC to the injury. In animals, deletion of a single allele of the TGF-β gene increases its susceptibility to endothelial cell activation and vascular lipid lesion formation in response to pro-atherogenic stimuli such as a lipid-rich diet[145]. TGF-β stimulates SMC proliferation at low concentrations *via* both PDGF-dependent and -independent manner. Ribozyme oligonucleotides against TGF-β increase vascular inflammation, accelerate lipid lesion formation, and shift the plaque morphology towards an unstable

phenotype[146]. Inhibition of TGF-β signaling in ApoE deficient mice, an animal model for atherosclerosis, suggest that the cytokine is critical for the production of extracellular matrix and the maintenance of a stable plaque phenotype through SMC phenotypic regulation[146,147]. Indeed, SMC in stable lesions express greater amounts of TGF-β than unstable lesions. MacCaffrey *et al*[148] demonstrate that SMCs isolated from atherosclerotic plaque tissue expressed less TβR Ⅱ than SMCs from healthy vessel wall. These data directly or indirectly show that TGF-β plays a pivotal role in the maintenance of normal blood vessel wall architecture.

Congenital heart diseases

Defective TGF-β signaling causes congenital heart diseases (CHD) during embryonic development[73]. CHD are the most commonly occurring birth defect in humans. Moderate and severe forms of congenital heart disease, including outflow tract defects and aortic arch anomalies, occur in 6 per 1000 live births[149]. Several studies have shown that cardiac neural crest contribute SMCs to the ascending and arch portions of the aorta and the ductus arteriosus. A number of congenital human diseases such as heart and outflow tract malformations are now attributed to failure of cardiac neural crests to differentiate into aortic arch complex[150]. Patients with Alagille syndrome always have CHD with right-sided outflow tract defects and tetralogy of Fallot influenced by cardiac neural crests. Patients with DiGeorge syndrome (DGS) always have congenital defects with heart and outflow tract malformations influenced by cardiac neural crest[151]. Mice with TβR Ⅱ mutation in neural crest develop all the morphological features of DGS. The hearts of TβR Ⅱ-mutant mice display a truncus arteriosus together with a ventricular septum defects (VSD) at E18. Both control and TβR Ⅱ-mutant neural crest cells are able to populate the pharyngeal apparatus and form aorto-pulmonary septum at E10.5. However, TβR Ⅱ-mutant neural crest cells in the aorto-pulmonary septum do not develop into smooth muscle cells[61]. The absence of neural crest derived SMCs in mutants explains the defective separation of the aorta from the pulmonary trunk, leading inevitably to a truncus arteriosus. Thus, TGF-β regulation of neural crest differentiation rather than migration plays a crucial role in the etiology of DiGeorge syndrome.

Thoracic aortic aneurysms and dissections

Combination of human molecular genetics and animal modeling has demonstrated the involvement of TGF-β signaling in aortic aneurysm[72]. Mutations in TβRI and TβR Ⅱ result in a spectrum of genetic conditions, associating with thoracic aortic aneurysms and dissections (TAAD)[152]. Mutations in TβR Ⅱ are initially identified in individuals with a Marfan-like connective tissue syndrome with TAAD and skeletal features of Marfan syndrome (MFS). TβRI and TβR Ⅱ mutations are subsequently described in individuals with Loeys-Dietz syndrome (LDS), a syndrome characterized by TAAD in children and young adults, arterial tortuosity, aneurysms and dissections of peripheral arteries. TβR Ⅱ mutations also lead to descending aortic disease and aneurysms of other arteries. An arginine residue of TβR Ⅱ at position 460 has been identified as a mutation "hot spot" for TAAD. Structural analysis has revealed that the amino acid substitutions may interfere with the receptor's ability to transduce signals[153]. In aortic SMCs explanted from patients with TβR Ⅱ mutations, the expression of SMC contractile proteins is decreased compared with controls. In vivo expression of contractile proteins is also decreased in aortas from patients with TβR Ⅱ mutations relative to unaffected aortas. The failed expression of SMC contractile proteins in TβR Ⅱ-mutant SMCs may influence the contractile function of SMCs, which contributes to the pathogenesis of TAAD[154].

Hypertension

Hypertension is defined as a sustained diastolic pressure of > 90 mmHg or a systolic blood pressure > 140 mmHg. Hypertension is another disease that related to SMC phenotypic switching. Although the etiology is extremely complex and varies among individuals, a common feature in the majority of cases of hypertension is an increase in peripheral resistance as a result of increased SMC contractility and vascular remodeling that are related with the phenotypic switching of SMC[155]. Primary pulmonary hypertension (PPH) is a rare disease with symptoms of fatigue, anorexia, an increase in pulmonary arterial pressure, right ventricular failure and death[156]. PPH is caused by mutations in either of two genes: the BMP type Ⅱ receptor gene (BMPR-Ⅱ) and ALK-1[157-159]. BMPR-Ⅱ mutations increase the incidence of PPH. BMP-2, -4 and -7 have been shown to inhibit SMC proliferation but increase the SMC marker expression in cultured pulmonary artery SMCs (PASMCs). PASMCs derived from the pulmonary arteries of patients with PPH exhibit abnormal growth responses to TGF-β. TGF- β inhibits serum-induced proliferation of PASMC from healthy individuals while stimulates cell proliferation of PASMCs from patients with PPH[160].

Hereditary hemorrhagic telangiectasia

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant vascular disorder which always happened in nasal, mucocutaneous, gastrointestinal, pulmonary, cerebral, and hepatic vascular beds. The common syndromes are nose bleeding, skin telangiectases, gastrointestinal bleeding[161]. Pulmonary arteriovenous malformations (PAVM) are always present in 20% of HHT populations. Genetic analyses reveal that Endoglin are responsible for type I HHT[162,163]. As aforementioned, Endoglin form TGF-β receptor complexes with TβRI and TβR Ⅱ to modulate the phosphorylation of TβRI and TβR Ⅱ and plays a pivotal role in angiogenesis as demonstrated in the $Eng^{-/-}$ mice. $Eng^{-/-}$ embryos show a defective development of VSMCs because of the reduced availability of active TGF-β protein. The lack of TGF-β inhibits

the recruitment and the differentiation of mesenchymal cells into VSMCs, leading to weak vessel walls, which may contribute to the development of HHT[76]. In addition to Endoglin, ALK-1 and Smad4 are also involved in HHT[164]. ALK-1 gene heterozygous mutation causes type Ⅱ HHT-2[164]. Recently, a remarkable ALK1 germinal and somatic mosaicism characterized by the presence of two distinct mutant alleles and a non-mutant ALK1 allele are identified in a woman with HHT and PAH[70]. It is proposed that genetic background and/or environmental factors (second hits), in addition to the mutations in Endoglin and ALK-1 genes, may also play an important role in the development of vascular malformations in HHT patients. Park and colleagues demonstrate using ALK-1-knockout mice that excisional skin wounding, as a second hit, is essential for the development of AVMs in HHT. These results provide new insights for understanding the pathogenesis of HHT[71].

CONCLUSION

SMC differentiation and phenotypic modulation play critical roles in embryonic cardiovascular development as well as pathological conditions in adults. TGF-β and its downstream signaling molecules including receptors, coreceptors and intermediate Smad proteins are all indispensible for the SMC differentiation or phenotypic modulation. As aforementioned, TGF-β itself can activate multiple signaling pathways such as MAPK, PI3K and RhoA. In addition, TGF-β signaling crosstalks with other pathways including Notch and SRF/myocardin. These diversified interactions ensure a precise cell fate determination and maturation of SMCs. Among the various SMC regulators, TGF-β/Smad signaling appears to be critical in regulating the initiation of SMC differentiation. Defective TGF-β signaling leads to development of several prominent cardiovascular diseases including congenital heart diseases, aortic aneurysm, hypertension, neointimal hyperplasia observed in vascular injury and atherosclerosis, *etc*.

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BRIEF ARTICLE

Biochemical analysis of the interactions of IQGAP1 C-terminal domain with CDC42

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Abstract

AIM: To understand the interaction of human IQGAP1 and CDC42, especially the effects of phosphorylation and a cancer-associated mutation.

METHODS: Recombinant CDC42 and a novel C-terminal fragment of IQGAP1 were expressed in, and purified from, *Escherichia coli*. Site directed mutagenesis was used to create coding sequences for three phosphomimicking variants (S1441E, S1443D and S1441E/S1443D) and to recapitulate a cancer-associated mutation (M1231I). These variant proteins were also expressed and purified. Protein-protein crosslinking using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide was used to investigate interactions between the C-terminal fragment and CDC42. These interactions were quantified using surface plasmon resonance measurements. Molecular modelling was employed to make predictions about changes to the structure and flexibility of the protein which occur in the cancer-associated variant.

RESULTS: The novel, C-terminal region of human IQGAP1 (residues 877-1558) is soluble following expression and purification. It is also capable of binding to CDC42, as judged by crosslinking experiments. Interaction appears to be strongest in the presence of added GTP. The three phosphomimicking mutants had different affinities for CDC42. S1441E had an approximately 200-fold reduction in affinity compared to wild type. This was caused largely by a dramatic reduction in the association rate constant. In contrast, both S1443D and the double variant S1441E/S1443D had similar affinities to the wild type. The cancer-associated variant, M1231I, also had a similar affinity to wild type. However, in the case of this variant, both the association and dissociation rate constants were reduced approximately 10-fold. Molecular modelling of the M1231I variant, based on the published crystal structure of part of the C-terminal region, revealed no gross structural changes compared to wild type (root mean square deviation of 0.564 Å over 5556 equivalent atoms). However, predictions of the flexibility of the polypeptide backbone suggested that some regions of the variant protein had greatly increased rigidity compared to wild type. One such region is a loop linking the proposed CDC42 binding site with the helix containing the altered residue. It is suggested that this increase in rigidity is responsible for the observed changes in association and dissociation rate constants.

CONCLUSION: The consequences of introducing negative charge at Ser-1441 or Ser-1443 in IQGAP1 are different. The cancer-associated variant M1231I exerts its effects partly by rigidifying the protein.

Key words: CDC42; Cytoskeleton; Protein phosphorylation;

Cancer-associated mutation; Protein-protein interaction

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INTRODUCTION

The IQGAP family of proteins function at the interface between cellular signalling and the cytoskeleton[1-3]. They receive information from a variety of signalling molecules, including kinases, small GTPases, growth factor receptors and calcium sensors[4-21]. This information is relayed directly to the actin cytoskeleton through interaction with filamentous actin (F-actin) which promotes filament bundling and caps the "barbed ends" of the filaments[22-25]. There are also indirect influences on the actin cytoskeleton mediated through the Wiskott-Aldrich Syndrome Protein (WASP) family[24,26] and with microtubules mediated via cytoplasmic linker protein 170 (CLIP-170) and adenomatous polyposis coli (APC) protein[27,28]. The IQGAP proteins are named after two key regions within them-the calmodulin binding IQ-motifs and GTPase activating protein (GAP) related domain (GRD). Although the GRD does bind the small GTPases CDC42 and Rac1[4], it does not function as the GTPase activator. Indeed, the available evidence suggests that it inhibits the catalytic activity of small GTPases[4,7]. This is consistent with sequence data and structural predictions. GAPs function by inserting an "arginine finger" into the active site of small GTPases which acts as a proton donor in the enzymatic mechanism of GTP hydrolysis[29-31]. IQGAPs lack this arginine residue and are thus not expected to be able to enhance the rate of GTP hydrolysis.

Humans have three IQGAP isoforms, IQGAP1, IQGAP2 and IQGAP3 with the first of these being the best characterised[32]. Like family members from other species they share a common domain organisation in which the actin-binding calponin homology domain (CHD) is at the N-terminus of the protein, the IQ-motifs are approximately in the middle of the primary sequence and the GRD is towards the C-terminus (Figure 1). There is no complete, three-dimensional structure of an IQGAP available, but it is assumed that the various domains fold in such a way to enable communication between them. It is also anticipated that there is considerable capacity for conformational change in the molecule in order to receive, integrate, interpret and output signals. The structures of some isolated domains have been determined. The structure of the CHD from human IQGAP1 has been solved by NMR spectroscopy and an x-ray structure of part of the GRD is also available[33,34]. Molecular modelling has predicted largely α-helical structures for the IQ-motifs[16,35].

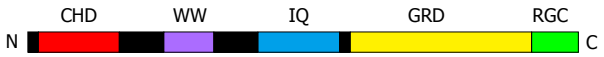

Figure 1 A schematic representation of human IQGAP1. The domains are shown on a linear representation of the protein sequence. The folded protein is unlikely to be arranged in a linear fashion and it is possible that some of these domains contact each other. CHD: Calponin homology domain (residues 44-159); WW: WW domain (679-712); IQ: IQ-motifs containing region (745-864); GRD: GAP-related domain (1004-1237); RGC: Ras-GAP C-terminal domain (1563-1657). The definitions of the domain boundaries are those of Briggs and Sacks[2].

In vitro biochemical studies on IQGAPs have tended to rely on fragments of the protein which are amenable to recombinant expression and purification in bacterial systems. CDC42 and Rac1 interaction with the GRD is promoted by the presence of GTP[4]. Phosphorylation of human IQGAP1 at Ser-1443, however, promotes interaction with CDC42 in the absence of nucleotide[36]. This phosphorylation, along with one at Ser-1441, promotes outgrowth of neurites[37].

Given the protein's involvement in the transduction of information from signalling pathways to the cytoskeleton, it is not surprising that it has been implicated in various types of cancer[38,39]. However, only one cancer-associated mutation in the coding sequence of the *Iqgap1* gene has been identified; this mutation results in the amino acid change M1231I[40]. It is not clear how this change affects the function of IQGAP1, although it does lie in the GRD prompting the hypothesis that it interferes with GTPase binding. However, this has not been tested experimentally.

Here, we identified a novel, biochemically amenable fragment from the C-terminal region of human IQGAP1 and confirmed that it is active, as judged by it ability to bind CDC42 in a crosslinking experiment. We then describe a detailed, quantitative investigation into the affinity of this interaction in the absence of added GTP. To probe the molecular consequences of phosphorylation in this region we used "phosphomicking" variants in which serine residues are replaced with negatively charged ones. We also recapitulated the cancer-associated variant M1231I in order to investigate its binding properties and carried out molecular modelling studies to provide further understanding the consequences of this alteration.

MATERIALS AND METHODS

Expression and purification of wild type and variant human IQGAP C-terminal region

The sequence encoding amino acids 877-1558 in human IQGAP1 was amplified by polymerase chain reaction

(PCR) using the Kazusa cDNA clone KIAA0051[41] as a template. The sequence was inserted into the pET-46 Ek/LIC (Merck, Nottingham, United Kingdom) by ligation independent cloning according to the manufacturer's instructions. Insertion into this vector introduces sequence encoding the amino acids MAHHHHHHVDDDDK at the 5'-end of the coding sequence. The complete coding sequence was verified (MWG Biotech, Ebersburg, Germany). The plasmid was transformed in to competent *Escherichia coli* (*E. coli*) HMS174(DE3). Colonies resulting from these transformations were picked and grown, shaking at 37 ℃, overnight in 5 mL of Luria-Bertani medium supplemented with 100 μg/mL of ampicillin. This overnight culture was diluted in to 1 L of Luria- Bertani medium supplemented with 100 μg/mL of ampicillin and grown, shaking at 37 ℃ until the cell density, as estimated by the $A_{600\ nm}$ reached 0.6 to 1.0 (typically 3-4 h). The culture was the induced by the addition of 1 mmol/L IPTG and grown for a further 3 h. Cells were harvested by centrifugation (4200 *g* for 15 min), resuspended in 20 mL of buffer R [50 mmol/L Hepes-OH, pH 7.5, 150 mmol/L sodium chloride, 10 %(v/v) glycerol] and stored, frozen at -80 ℃ until required.

These cell suspensions were thawed and then disrupted by sonication (three 30 s pulses of 100 W, with 30-60 s gaps in between to allow cooling of the cells). Cell debris was removed by centrifugation (20 000 *g* for 15 min) and the supernatant applied to a 1 mL nickel-agarose column (His-Select, Sigma, Poole, United Kingdom) which had been previously equilibrated in buffer A [50 mmol/L Hepes-OH, pH 7.5, 500 mmol/L sodium chloride, 10 %(v/v) glycerol]. The cell extract was allowed to pass through the column by gravity flow and the column was washed with 20 mL of buffer A. Protein was eluted with three 2 mL washes of buffer B (buffer A supplemented with 250 mmol/L imidazole). Protein containing fractions were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and dialysed overnight at 4 ℃ against buffer D (buffer R supplemented with 2 mmol/L dithiothreitol). The protein concentration was determined by the method of Bradford[42], using bovine serum albumin as a standard. Aliquots (50-100 μL) of the protein were stored frozen at -80 ℃.

Mutations were introduced in to the coding sequence using the "QuikChange" protocol[43] and verified by DNA sequencing. Each variant protein was expressed in, and purified from, *E. coli* using essentially the same procedure as outlined above for the wild type.

Expression and purification of human CDC42

The complete coding sequence of human CDC42 was amplified by PCR using IMAGE clone 3626647[44] as a template and inserted into pET-46 Ek/LIC. The DNA sequence of the insert was verified. The expression and purification of the protein was carried out using the same protocol as for IQGAP1-CTD.

Crosslinking of CDC42 and IQGAP

GTP bound CDC42 was prepared by incubating a mixtures of 6 μmol/L CDC42, 0.9 mmol/L GTP and 0.9 magnesium chloride on ice for 30 min. Nucleotide-depleted (ND) CDC42 was prepared by incubating 6 μmol/L CDC42 with 5 mmol/L EDTA on ice for 30 min. Protein-protein crosslinking was carried out using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). Methods were based on those previously used for the detection of interaction between the atrial myosin essential light chain and F-actin[45,46]. Untreated, GTP-loaded or ND CDC42 (3 μmol/L) was mixed with IQGAP(DR6) (3 μmol/L) and incubated for 30 min at 22 ℃. EDC was then added to a final concentration of 0.6 mmol/L and the incubation continued for a further 60 min. Products were analysed by SDS-PAGE.

Surface plasmon resonance

Surface plasmon resonance was measured using a BIAcore 3000 instrument (BIAcore, Uppsala, Sweden). Prior to analysis all proteins were dialysed into HBS Buffer (BIAcore; 10 mmol/L Hepes, pH 7.4, 150 mmol/L NaCl). CDC42 was immobilised onto a CM5 sensor chip (BIAcore) using N-hydroxysuccinimide (NHS)/EDC chemistry. The surface was activated with a mixture of 100 mmol/L NHS and 400 mmol/L EDC for 30 min. CDC42 (25 μmol/L) was then flowed over the surface for two 7 min periods and the surface was then blocked and deactivated with 1 M ethanolamine for 30 min. Immobilisation of CDC42 resulted in a change in the response units (RU) of approximately 1400 RU.

Binding was measured by flowing 0.5 μmol/L to 2.5 μmol/L IQGAP(DR6) over the surface for 300 s (association phase) followed by buffer for 300 s (dissociation phase). In between binding measurements, the surface was regenerated by the injection of sodium hydroxide (5 mmol/L for 220 s). For each binding measurements controls were carried out in parallel in which the protein was flowed over a cell which had been activated with NHS/EDC and blocked with ethanolamine. To determine the response due to interaction between IQGAP(DR6) and CDC42, the readings for the controls were subtracted from the experimental ones. The association and dissociation rate constants (k_a and k_d, respectively) and the dissociation equilibrium constant (K_D) were determined by non-linear curve fitting of the data using BIAevaluation software.

Molecular modelling

The structure of human IQGAP1, residues 962-1345 (PDB 3FAY)[34], was taken as a starting point for molecular modelling studies. This structure file describes one, unbroken polypeptide chain. The selenomethionine residues in this structure were altered to methionine using PyMol (www.pymol.org) and the resulting structure energy minimised using YASARA[47]. Residue 1231 in this minimised structure was altered to isoleucine, and the mutated structure

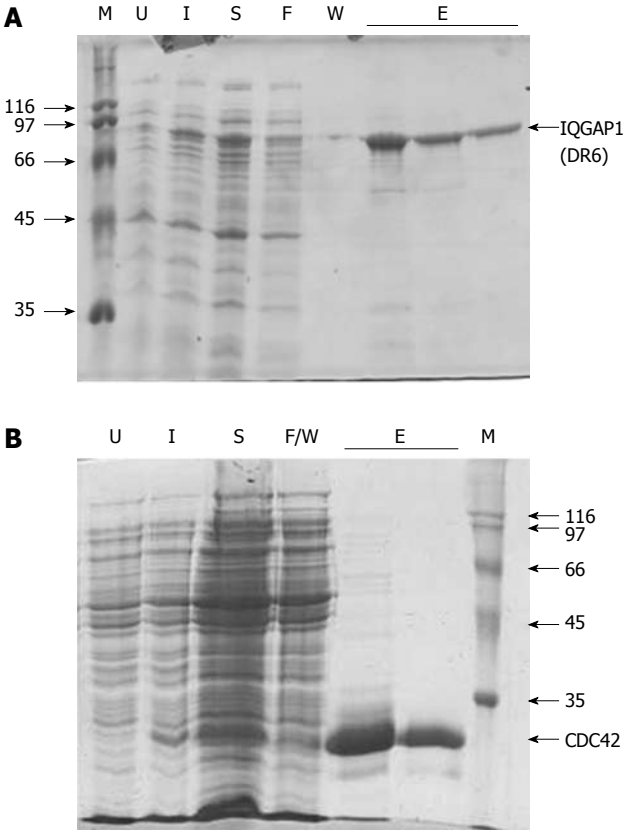

Figure 2 Proteins used in this study. The recombinant expression and purification of (A) IQGAP1(DR6) and (B) CDC42 monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In both (A) and (B), U, extract from cells prior to induction; I, extract from cells 2-3 h after induction; S, soluble material remaining after sonication; F, material which flowed through the column without binding; W, material removed in the washing steps; E, elutions; M, molecular mass markers (with their masses shown to the side of the gel in kDa). In the case of CDC42, F and W were combined into a single sample.

re-minimised using YASARA. Polypeptide flexibility was estimated by generating 500 conformers in the momentum motion type mode of FIRST/FRODAN with an energy cut off of -1 kcal/mol[48,49].

RESULTS

Identification of a biochemically tractable C-terminal fragment of human IQGAP1

Previous reports demonstrated that a fragment beginning at residue 864 and continuing through to the C-terminus of the protein (residue 1657) can be expressed in, and purified from, *E. coli*, albeit at relatively low levels[4]. We noted that the structure of the Ras-GAP C-terminal domain (RGD), a 112 amino acid residue region at the extreme C-terminus of the protein has been deposited in the Protein Data Bank (PDB ID: 1X0H). From this we reasoned that there must be a domain boundary in the region of residue 1545. Therefore, a region beginning at residue 877 and finishing at 1558 was expressed. This fragment, which we named IQGAP1(DR6), can be purified with good yield, typically 1-2 mg per litre of *E. coli* culture (Figure 2A). Similar purities and yields were achieved with the various variant

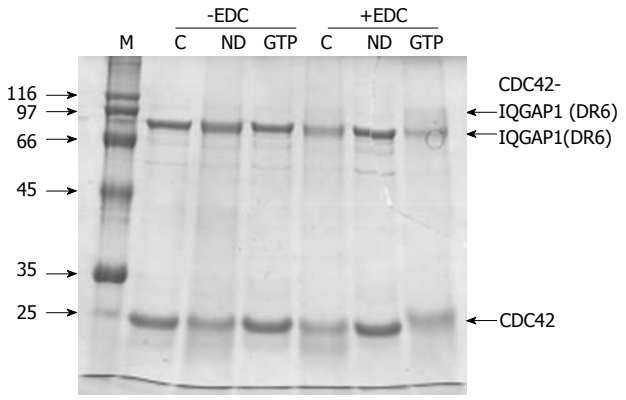

Figure 3 Interaction between IQGAP1 and CDC42. An interaction between IQGAP1(DR6) and CDC42 can be detected by EDC crosslinking in the presence of GTP. In the absence of EDC (lanes headed -EDC), a mixture of IQGAP(DR6) and CDC42 (lane C) behaves the same on SDS-PAGE as IQGAP1(DR6) and nucleotide-depleted CDC42 (lane ND) and as IQGAP1(DR6) and GTP-loaded CDC42 (lane GTP). In the presence of the crosslinker (lanes headed +EDC), crosslinking was not observed between IQGAP(DR6) and CDC42 (C) or IQGAP(DR6) and nucleotide-depleted CDC42 (ND). However an additional band, corresponding to approximately the combined molecular masses of IQGAP(DR6) and CDC42 is seen with GTP-loaded CDC42 (GTP).

proteins also described in this work (data not shown). Full length, recombinant, human CDC42 could also be purified in good yield (Figure 2B).

Interactions between CDC42 and IQGAP1(DR6)

Recombinant, human CDC42 was shown to interact with the C-terminal domain fragment. The two proteins could be cross-linked using the reagent EDC which is specific to carboxylate and amino groups (Figure 3). This demonstrates that the recombinant C-terminal fragment is likely to be folded and is functional. The amount of crosslinked product was greatest in the presence of GTP (Figure 3).

Effects of phosphomimicking mutations

To investigate the effects of phosphorylation at serines 1441 and 1443, the phosphomimic variants S1441E, S1443D and S1441E/S1443D were constructed. These amino acid changes insert negative charges into the structure at the sites which can be phosphorylated. Similar mutants have been shown to recapitulate the effects of phosphorylation in an *in vivo* cell model[37]. Since it has been hypothesised that phosphorylation increases the affinity for CDC42 in the absence of GTP[36], this interaction was investigated by surface plasmon resonance. Interaction between the wild type and immobilised CDC42 could be detected by surface plasmon resonance in the absence of added GTP (Figure 4). Fitting of these data resulted in rate constants for the association and dissociation phases of the reactions (k_a and k_d) and, consequently, a value for the dissociation constant (K_D) (Table 1). It was noted that these fits were not perfect with some non-random residuals (not shown). This may indicate that there is heterogeneity in the preparations and/or that the binding event is more complex. However, for the purposes of comparison, the simple bimolecular interaction model was used

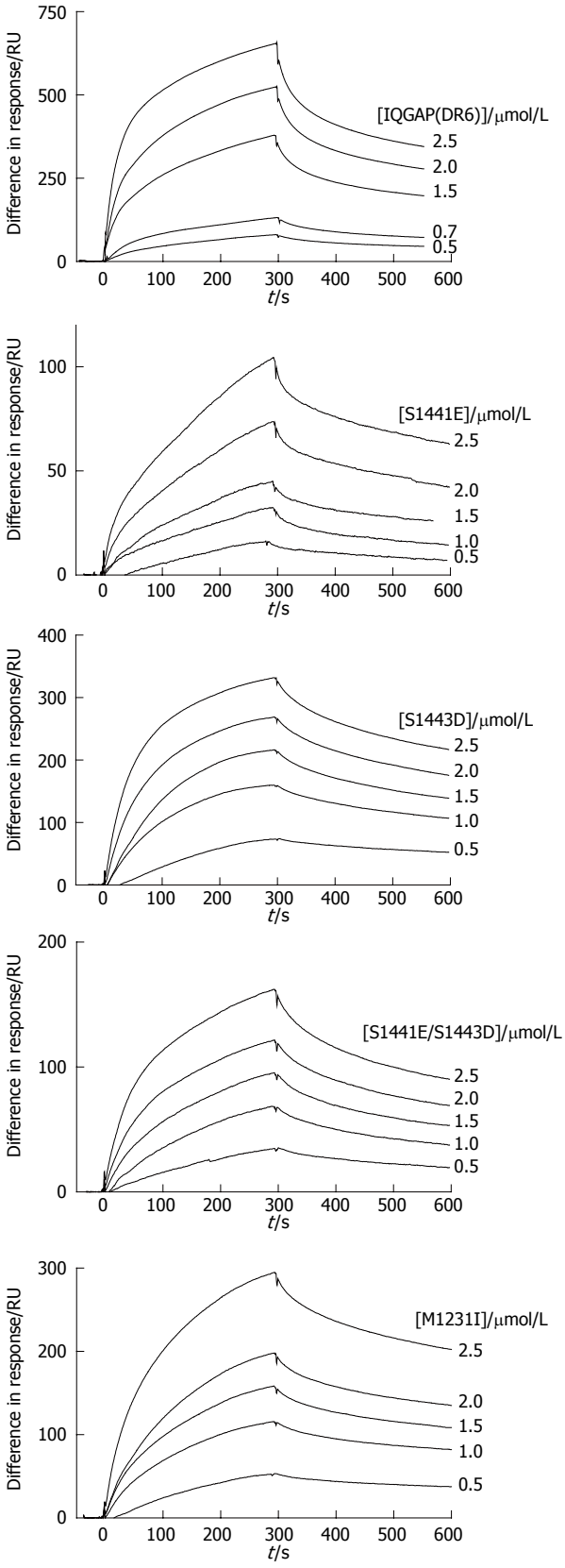

Figure 4 Interaction of IQGAP-CTD and CDC42 can be detected by surface plasmon resonance. Typical sensorgrams resulting from flowing IQGAP1(DR6) wild type and variants over immobilised CDC42 (for conditions, see Materials and Methods). From top to bottom, wild type, S1441E, S1443D, S1441E/S1443D, M1231I. Protein concentrations are shown to the right of the sensorgrams.

Table 1 Binding parameters for the interaction of IQGAP1(DR6) and CDC42

IQGAP1(DR6) variant	k_a/l.mol^{-1}.s^{-1}	k_d/s^{-1}	K_D/μmol/L
Wild type	4900 ± 100	(6.5 ± 0.5) × 10^{-3}	1.3 ± 0.13
S1441E	12 ± 1	(2.7 ± 0.03) × 10^{-3}	220 ± 21
S1443D	5800 ± 100	(4.7 ± 0.2) × 10^{-3}	0.81 ± 0.048
S1441E/S1443D	3600 ± 100	(4.0 ± 0.2) × 10^{-3}	1.1 ± 0.086
M1231I	1800 ± 100	(1.7 ± 0.2) × 10^{-3}	0.90 ± 0.16

These were determined by surface plasmon resonance. The values are reported ± their standard errors as determined by the BIAevaluation fitting programme (see Materials and Methods).

throughout.

All three phosphomimic variants also bound to CDC42 in the absence of additional nucleotide. However, in the case of S1441E, the affinity was reduced by two orders of magnitude. This arises mainly because of a reduction in the association rate constant. It should be noted that this reduced value (12 l.mol^{-1}.s^{-1}) is very low and, therefore, may be subject to greater error than the other values. Interestingly, the double mutant (S1441E/S1443D) binds with a similar affinity to the wild type (Table 1).

Effects of the cancer-associated mutation, M1231I

The ability of the disease-associated variant to interact with CDC42 was tested by surface plasmon resonance. These experiments suggest that it is able to do so with similar affinity to the wild type protein. However, both the association and dissociation rate constants are reduced compared to wild type (Table 1).

To help understand the biochemistry of the M1231I variant protein, a molecular model was constructed based on the crystal structure of the GRD. This suggested that the overall fold is not greatly changed by the substitution of this methionine for isoleucine (rmsd between the wild type and variant protein 0.564 Å over 5556 equivalent atoms). The residue lies towards the surface of the protein, away from the predicted GTPase binding site. In addition to gross structural changes, the functions of proteins can be affected by the flexibility of the molecule[50]. Computational estimation of the backbone flexibility of the molecule suggested that the M1231I variation results in changes in flexibility at a number of sites within the protein (Figure 5A). The site with the greatest loss of flexibility is a loop (Ser-1212 to Leu-1217) which links the α-helix containing residue 1231 with residues predicted to play a key role in the CDC42 binding site (Tyr-1192 to Arg-1194; Figure 5B). This loss of flexibility may affect the dynamics of small GTPase interaction.

DISCUSSION

These experiments establish a new fragment from the C-terminal region of human IQGAP1 which is amenable to biochemical analysis. The fragment interacts with CDC42, and the strength of interaction is increased in

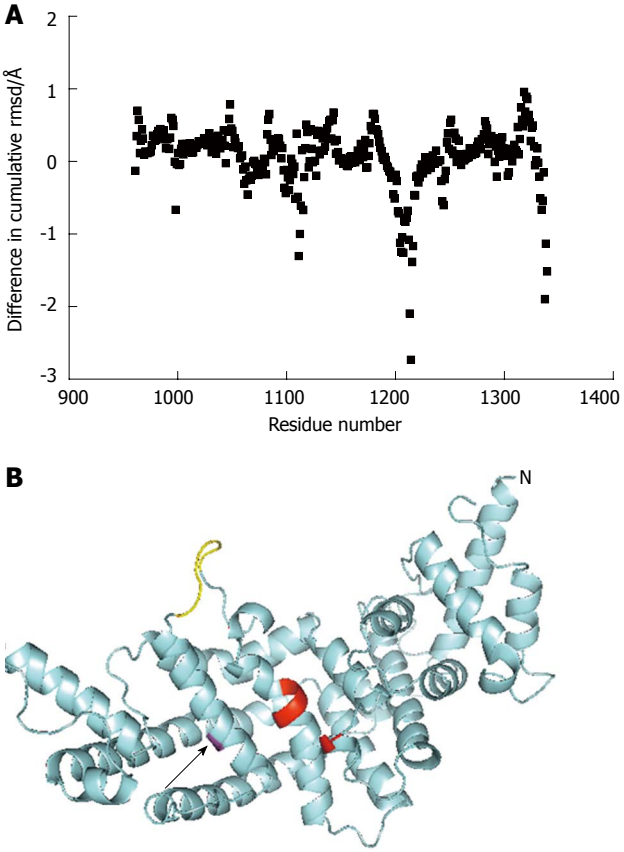

Figure 5 Predicted structural consequences of the M1231I, cancer associated, mutation. A: The predicted effects on backbone flexibility as determined by FIRST/FRODAN (see Materials and Methods) are plotted as the difference in cumulative flexibility between the M1231I variant and wild type. Thus, a negative value represents in loss in flexibility of the variant compared to wild type. The greatest predicted rigidification occurs around residue 1214 and is indicated with an arrow; B: A model of the GAP-related domain of the M1231I variant is shown in cyan, with Ile-1231 in magenta and indicated with an arrow. The N-terminus of the fragment is marked (N) and the C-terminus can be seen close to this in space. Key residues predicted to be involved in CDC42 interaction (Thr-1046 and Tyr-1192 to Arg-1194) are shown in red. A loop (Ser-1212 to Leu-1217) predicted to be more rigid in the variant compared to the wild type is shown in yellow. It links the helix containing residue 1231 and part of the CDC42 binding site.

the presence of GTP. Interestingly, previous work has demonstrated, using isothermal titration calorimetry, an interaction between a C-terminal fragment of IQGAP (residues 962-1345) and GTP-loaded CDC42, but not with CDC42 purified in the absence of added nucleotides (assumed to be GDP-loaded)[34]. This may indicate that the additional residues present in the IQGAP1(DR6) fragment are important in CDC42 interaction in the absence of GTP. The phosphomimicking variants suggest that phosphorylation of the two serine residues has different effects. While the S1443D variant has slightly increased affinity for CDC42, the affinity of S1441E is decreased and introduction of a negative charge at both sites restores the affinity to essentially wild type levels. This suggests that there may be crosstalk between the two serines within the C-terminal domain. The results from the cancer-associated variant emphasise the importance of considering changes in protein flexibility, as well as changes to overall structure.

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COMMENTS

Background

IQGAP family proteins act as "molecular telephone exchanges" collecting information from a variety of cellular signalling pathways, integrating this information and passing it on to the cytoskeleton. They are multi-domain proteins and biochemical studies have tended to concentrate on elucidating the roles of the individual domains. Given their role in organising the cytoskeleton in response to cellular signalling, it is not surprising that IQGAPs are implicated in a number of diseases, including bacterial infections and cancers.

Research frontiers

Human IQGAP1's activity can be regulated by phosphorylation at serines 1441 and 1443. There is also a cancer-associated mutation, M1231I. All these residues lie in the C-terminal, GTPase-binding region of the protein. The molecular consequences of these changes on the affinity of IQGAP1 for CDC42 have not been investigated in detail previously.

Innovations and breakthroughs

We demonstrate that phosphomimicking alterations at residues 1441 and 1443 have different effects on the affinity for CDC42. The M1231I variant has similar affinity for wild type, but the association and dissociation rate constants are both reduced. Molecular modelling suggests that this variant does not cause any significant structural changes to the protein, but that it does reduce mobility in a key loop which links the residue 1231 to the putative CDC42 binding site.

Applications

The biochemically amenable, functional fragment of human IQGAP1 which we identify will have application in further biochemical studies on this protein. If further cancer-associated mutations are discovered in *Iqgap1* it will be interesting to compare results and see if these also alter the flexibility of the protein.

Terminology

Cancer-associated mutation: A mutation in the gene sequence encoding a protein which is associated with a higher risk of cancer. Care should be taken not to assume that the mutation causes cancer. CDC42: A small GTPase involved in regulating the cytoskeleton in eukaryotic cells; Crosslinking: A method for detecting interactions between proteins. It is especially useful for capturing short-lived, transient interactions (which are often found in signalling complexes); IQGAP: A family of cytoskeletal scaffolding proteins. Humans have three-IQGAP1, IQGAP2 and IQGAP3.

Peer review

This manuscript is worthy of publishing because the contents do accord with the Journal scopes and the biochemical characterization provides insight to understand IQGAP/CDC43 interaction. The technique is sound; but the discussion is not thorough.

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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