DRAGON, a GPI-anchored membrane protein, inhibits BMP signaling in C2C12 myoblasts

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Bone morphogenetic proteins (BMPs) induce osteoblastic differentiation of myoblasts via binding to cell surface receptors. Repulsive guidance molecules (RGMs) have been identified as BMP co-receptors. We report here that DRAGON/RGMb, a member of the RGM family, suppressed BMP signaling in C2C12 myoblasts via a novel mechanism. All RGMs were expressed in C2C12 cells that were differentiated into myocytes and osteoblastic cells, but RGMc was not detected in immature cells. In C2C12 cells, only DRAGON suppressed ALP and Id1 promoter activities induced by BMP-4 or by constitutively activated BMP type I receptors. This inhibition by DRAGON was dependent on the secretory form of the von Willebrand factor type D domain. DRAGON even suppressed BMP signaling induced by constitutively activated Smad1. Over-expression of neogenin did not alter the inhibitory capacity of DRAGON. Taken together, these findings indicate that DRAGON may be an inhibitor of BMP signaling in C2C12 myoblasts. We also suggest that a novel molecule(s) expressed on the cell membrane may mediate the signal transduction of DRAGON in order to suppress BMP signaling in C2C12 myoblasts.

Introduction

Bone morphogenetic proteins (BMPs) were first isolated from demineralized bone matrix and identified as factors responsible for inducing ectopic bone formation in muscular tissues (Urist 1965; Wozney et al. 1988). BMPs are responsible not only for artificial ectopic bone formation but also for physiological skeletal development (Thomas et al. 1997). BMP signaling is initiated when a ligand binds to complexes of type I and type II serine/threonine kinase receptors (Miyazono et al. 2005; Katagiri et al. 2008). The BMP-bound type II receptor phosphorylates the intracellular “GS” domain of the type I receptor. The activated type I receptor then phosphorylates Smad1/5/8 transcription factors in the cytoplasm. The phosphorylated Smad1/5/8 form transcriptionally active complexes with Smad4, translocate into the nucleus and bind to the regulatory elements of target genes (including Id1, which encodes an inhibitor of myogenesis) (Afrakhte et al. 1998; Katagiri et al. 2008). Among the type I receptors, substitution mutations of the specific conserved residues in the GS domains activate intracellular signaling without the binding of ligands or type II receptors (Wieser et al. 1995; Akiyama et al. 1997; Fujii et al. 1999; Aoki et al. 2001). Recently, we found that substitution of the phosphorylation site at the carboxyl terminus in Smad1 also activated downstream signaling events, including Id1 expression and osteoblastic differentiation, without phosphorylation by BMP type I receptors (Nojima & Katagiri, unpublished observation).

The repulsive guidance molecule (RGM) family members RGMa, DRAGON/RGMb and RGMc/hemojuvelin/HEF2 are secretory glycosylphosphatidylinositol (GPI)-anchored membrane proteins that have been implicated in axonal guidance and neural tube formation during embryogenesis (Monnier et al. 2002; Niederkofler et al. 2004; Papanikolaou et al. 2004; Samad et al. 2004). They have significant sequence homology to one another (50–60% amino acid identity) (Papanikolaou et al. 2004;
willbrand factor (vWF) type D domain (Monnier et al. 2002), although its physiological function is still unknown. All of the RGM family members were found to act as co-receptors for the BMP subfamily. These proteins bind to BMP ligands as well as type I and type II BMP receptors, thereby enhancing BMP signaling (Babitt et al. 2005, 2006; Samad et al. 2005). A genetic mutation was found in the hemojuvelin gene in patients with juvenile hemochromatosis, a condition that is caused by the decreased expression of hepcidin, a key regulator of iron homeostasis in hepatocytes. Hemojuvelin enhanced the BMP-dependent up-regulation of hepcidin expression in hepatocytes (Papanikolaou et al. 2004; Babitt et al. 2006, 2007). We report here that DRAGON inhibits BMP signaling in C2C12 myoblasts via a region that includes the secretory form of the vWF type D domain. DRAGON also inhibits the BMP signaling induced by a constitutively active form of Smad1. These findings indicate that a novel molecule(s) expressed on the cell membrane may mediate the signal transduction of DRAGON to suppress BMP signaling in C2C12 myoblasts.

Results
Expression levels of RGMs in C2C12 cells and mouse tissues
First, we examined the expression levels of RGM mRNAs in C2C12 myoblasts. RGMa and DRAGON were expressed in both growing and differentiated myocytes, but RGMc was detected only in myogenin-expressing differentiated cells (Fig. 1a). Over-expression of a constitutively activated BMP type I receptor also induces similar differentiation conversions of myoblasts (Katagiri et al. 1994). Over-expression of a constitutively activated BMP type I receptor also induces similar differentiation conversions of myoblasts (Katagiri et al. 1994). Over-expression of a constitutively activated BMP type I receptor also induces similar differentiation conversions of myoblasts (Katagiri et al. 1994). Over-expression of a constitutively activated BMP type I receptor also induces similar differentiation conversions of myoblasts (Katagiri et al. 1994). Over-expression of a constitutively activated BMP type I receptor also induces similar differentiation conversions of myoblasts (Katagiri et al. 1994). Over-expression of a constitutively activated BMP type I receptor also induces similar differentiation conversions of myoblasts (Katagiri et al. 1994).
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osteoblasts because it was not expressed in primary osteoblasts (Fig. 1b,c).

**DRAGON inhibits BMP signaling in C2C12 cells**

Next, we examined the effect of RGMs on BMP-induced osteoblastic differentiation. A constitutively activated BMP receptor—ALK2(R206H), ALK2(Q207D) or BMPR-IA(Q233D)—was co-transfected with one of the RGMs into C2C12 cells, and as a result of that process we identified alkaline phosphatase (ALP) activity as a typical marker of osteoblastic differentiation. Unexpectedly, DRAGON suppressed ALP activity in all of the cultures that were examined (Fig. 2a–c). The suppression of BMP signaling by DRAGON was also confirmed in a luciferase assay using BMP-specific IdWT4F-luc in C2C12 cells (Fig. 2d,e). In contrast to BMP signaling, TGF-β signaling (as determined by CAGA-luc) was not suppressed by DRAGON in C2C12 cells (Fig. 2f). DRAGON showed a weaker inhibitory activity on IdWT4F-luc induced by ALK2(Q207D) in HepG2 cells compared with the inhibitory activity seen in C2C12 cells (Fig. 2d,g).

**Deletion analysis of DRAGON to determine the inhibitory domain affecting BMP signaling**

We generated Sol- and Cyt-DRAGON mutants, in which a GPI-anchor domain and a signal peptide, respectively, were deleted (Fig. 3a). The cellular localization of these DRAGON proteins was confirmed by immunohistochemical analysis in the presence and absence of detergent. Wild-type DRAGON, but not Sol- or Cyt-DRAGON, was detected on the cell membrane, even in the absence of detergent; Sol- and Cyt-DRAGON were detected only in the cytoplasm in the presence of detergent (Fig. 3b). ALP activity inhibition induced by ALK2(Q207D) was observed in the wild-type and Sol-DRAGON constructs, which may be secreted by a signal peptide. To investigate whether the signal peptide itself suppressed ALP activity, we further generated an additional mutant of DRAGON (Swap-DRAGON) in which the signal peptide was replaced with that of another protein (Fig. 3a). Swap-DRAGON was anchored on the cell membrane and suppressed ALP activity in a manner similar to that of wild-type DRAGON, indicating that the signal peptide of
DRAGON was not involved in the inhibition of BMP signaling (Fig. 3b,c).

We further examined the inhibitory domain of DRAGON using the three deletion mutants 271, 321 and 354 (Fig. 4a). All of these deletion mutant DRAGON constructs were detected on the cell membrane in the absence of detergent (Fig. 4b). Both the 271 and 321 mutants showed the capacity to inhibit ALP activity, but mutant 354 had little suppressive effect on enzyme activity (Fig. 4c). Together, these findings indicate that a region including the vWF type D-like domain may play an important role in the inhibition of BMP signaling by DRAGON.

DRAGON inhibits BMP signaling independent of neogenin

Because the secretory form of DRAGON inhibited BMP signaling without a GPI-anchor domain (Figs 3, 4), we hypothesized that a cell surface molecule(s) may be involved in the inhibitory activity of DRAGON. Neogenin has been identified as a cell surface receptor for RGM family members (Matsunaga et al. 2004; Rajagopalan et al. 2004). Because neogenin was only weakly expressed in C2C12 cells, we cloned its cDNA and transiently over-expressed it in C2C12 cells (Fig. 5a, unpublished observation). Co-transfection of neogenin showed no effect on the ability...
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Both wild-type and Sol-DRAGON decreased the expression levels of ALP and osterix mRNAs induced by ALK2(Q207D) in C2C12 cells, suggesting that DRAGON inhibited BMP signaling-induced osteoblastic differentiation (Fig. 6a). We examined the effect of DRAGON on phosphorylation levels of Myc–Smad1 and found that DRAGON did not significantly decrease phospho-Smad1 levels (Fig. 6b). Next, we asked whether a direct interaction between DRAGON and BMP receptor is involved in the inhibition process. Immunoprecipitation with immunoblotting analysis indicated that wild-type DRAGON, but not Sol-DRAGON, interacted with ALK2(Q207D) (Fig. 6c). Finally, we examined the effect of DRAGON on constitutively activated Smad1, which is able to induce downstream signal transduction cooperatively with Smad4 without phosphorylation by BMP receptors. Wild-type DRAGON, Sol-DRAGON and mutant 321 markedly inhibited the ALP activity and IdWT4F–luc activity induced by constitutively activated Smad1 and Smad4 (Fig. 6d,e). These results indicate that the inhibitory effect of DRAGON represses the transcriptional activity of Smad. Because both wild-type DRAGON and Sol-DRAGON suppressed ALK2 activity to a similar degree, we hypothesized that the interaction between DRAGON and BMP receptors may not be involved in this inhibition.

Discussion

The present study demonstrates that DRAGON, a member of the RGM family, inhibits BMP signaling in C2C12 myoblasts. The inhibitory domain of DRAGON was mapped around the vWF type D-like (but not GPI-anchor) domain. In our preliminary experiments, mouse vWF did not have any effect on BMP signaling in C2C12 cells, suggesting that a DRAGON-specific sequence in this domain may be involved in this inhibition. Although RGMs have been reported to interact with both BMP ligands and BMP receptors, DRAGON suppressed signaling induced by constitutively activated BMP receptors in the absence of BMP ligands. Moreover, secretory forms of DRAGON suppressed BMP signaling induced by constitutively activated Smad1. Taken together, our results suggest that DRAGON may bind to a novel molecule(s) expressed on the cell membrane to transduce inhibitory signaling on the transcriptional activity of Smads (Fig. 6f).

Neogenin, a homologue of deleted colorectal cancer (DCC) and the netrin-1 receptor, was a potential candidate for the DRAGON binding protein on the cell membrane because it has been shown to act as a high-affinity receptor for RGMs (Vielmetter et al. 1994; Keino-Masu et al. 1996; Stein et al. 2001). The interactions between RGMa and neogenin were involved in the regulation of neuronal survival (Wilson & Key 2006). Interestingly, the interaction with neogenin was required for RGMc release from muscle cells, and soluble RGMc played a critical role in the negative regulation of hepatic hepcidin expression through BMP signaling in hepatocytes (Zhang et al. 2007). However, neogenin over-expression did not affect the DRAGON activity in C2C12 cells. We noticed that the degree of BMP signaling inhibition by DRAGON is dependent on cell type; for example, HepG2 and other types of cells showed weaker activities than C2C12 cells. It is possible that the amount of novel molecule(s) expressed on the cell membrane may regulate the cell type-specific activity of DRAGON. In addition, DRAGON was expressed in bone, cartilage and primary osteoblasts. However, the expression of DRAGON was unaltered during osteoblastic differentiation in C2C12 cells. Thus, it is possible that the suppressive function of DRAGON depended
on the alteration of expression levels of a novel molecule(s). The identification of the DRAGON–binding molecule(s) on the cell membrane will shed light on the novel regulatory mechanisms associated with BMP signaling.

Recently, heterozygous mutations in the ACVR1 gene, which encodes the BMP type I receptor ALK2, were identified in familial and sporadic patients with FOP (Shore et al. 2006; Nakajima et al. 2007; Fukuda et al. 2009). These mutations cause an amino acid substitution in the intracellular ALK2 domain. We found that these ALK2 mutants were constitutively activated BMP receptors and that they cooperatively induced BMP signaling and osteoblastic differentiation with Smad1 and Smad5 (Fukuda et al. 2009). At the present time, no treatments are available to prevent heterotopic bone formation in FOP. We found in this study that DRAGON clearly inhibited the activity of ALK2(R206H), a typical mutation in FOP patients. Therefore, DRAGON may provide a novel concept for intracellular signal transduction inhibition in the design of novel drugs for the treatment of FOP.
In conclusion, DRAGON inhibited the intracellular signaling of activated BMP receptors by suppressing the transcriptional activity of Smad in C2C12 cells. DRAGON may bind to a novel molecule(s) expressed on the cell surface to transduce the inhibitory signals of BMPs.

Experimental procedures

Cell culture, transfection, reporter assay and ALP assay

Mouse C2C12 myoblasts and human HepG2 hepatocytes were maintained as described (Katagiri et al. 1994; Babitt et al. 2006). Myogenic differentiation of C2C12 cells was induced by culturing the cells with differentiation medium (Dulbecco’s modified Eagle’s medium containing 2.5% fetal bovine serum) for 7 days. Cells were transfected with plasmids using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. BMP and TGF-β signaling were monitored using IdWT4F-luc and CAGA-luc reporter plasmids, respectively (Dennler et al. 1998; Katagiri et al. 2002). ALP activity was measured as described (Kodaira et al. 2006). In brief, cells were incubated with a substrate solution (0.1 m diethanolamine, 1 mM MgCl₂ and 10 mg/mL of p-nitrophenylphosphate). After appropriate incubation, reactions were terminated by adding 3 m NaOH; absorbance was measured at 405 nm.

Reverse transcription-PCR analysis and plasmid construction

Total RNAs were prepared using TRIzol Reagent (Invitrogen) and then reverse transcribed using Go-Taq (Promega, Madison, WI). The primer sets used were previously described (Zhao et al. 2002). A series of mutant DRAGON constructs was generated using a standard PCR technique.

Immunoblotting and immunohistochemistry

Cells were lysed in TNE buffer [10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA and 1% Nonidet P-40] and subjected to immunoprecipitation and immunoblotting as described previously (Fukuda et al. 2009). The following antibodies were used: anti-phosphorylated Smad1/5/8 antibody (Cell Signaling, Beverly, MA), anti-V5 antibody (Invitrogen), anti-FLAG antibody (Sigma, St Louis, MO), anti-Myc antibody and anti-actin antibody (SantaCruz, Santa Cruz, CA).

The cells transfected with DRAGON mutants were fixed with formalin and stained with an anti-FLAG antibody and DAPI.

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