Phosphotyrosine binding (PTB) domain-containing proteins are implicated in signal transduction, protein trafficking, and cytoskeletal dynamics (1). The PTB domain is important for protein-protein interactions that are either phosphotyrosine-dependent or -independent. As a result, PTB-containing proteins engage in a wide range of cellular functions (2). PTB domain proteins play a pivotal role in signal transduction mediated by various growth factors, including epidermal growth factor (3), insulin (4, 5), nerve growth factor (6), and transforming growth factor β (TGF-β) (7, 8). To mediate TGF-β signaling, PTB proteins such as Dab2 (7) and Dok1 (8) act as adaptors to stabilize the interaction between the receptor and Smad2/3.

TGF-β signaling is implicated in a number of cellular functions such as proliferation, migration, differentiation, and apoptosis. Disturbance of TGF-β signaling is related to serious human diseases including cancer, fibrosis, and heritable disorders (9). TGF-β signaling is initiated by the binding of ligands to two types of Ser/Thr kinase receptors, called type I and type II receptors. The type I receptor is activated by the type II receptor and propagates intracellular signaling to the nucleus through the phosphorylation of receptor-activated Smads (R-Smad) including Smad1, -2, -3, -5, and -8. Then, the active Smad complex that is formed by the interaction of R-Smad with Smad4 translocates into the nucleus where it regulates the transcription of target genes.

TGF-β signaling is also crucial for developmental processes including germ-layer specification and patterning during embryogenesis. *Xenopus* late blastula embryo consists of three regions with different cell fates including the animal cap, the marginal zone, and the vegetal mass (10). As development proceeds, animal cap cells become ectodermal derivatives such as the skin and nervous system, and marginal zone cells develop into mesodermal derivatives such as bone, blood, kidney, and muscle. Vegetal cells grow into endodermal derivatives such as lung, pancreas, and digestive organs. In *Xenopus* early embryo, the mesodermal cell fate is established in the marginal zone between the animal and vegetal poles by inductive signals from the underlying endoderm. Several TGF-β family ligands, such as activin, Vg1, and nodal-related proteins are responsible for the induction of mesoderm and endoderm, as well as the subsequent embryonic patterning.

In this study, we identified a novel PTB protein, Xdpcp (*Xenopus* dok-PTB containing protein), as a negative regulator of activin/nodal signaling, a branch of TGF-β superfamily signaling. Activin/nodal signaling is essential for mesoderm induction and patterning in *Xenopus* embryos (11). We show that Xdpcp negatively regulates the activin/nodal signaling by inhibiting the interaction between the activin receptor and Smad2, and plays a pivotal role in the regulation of mesendoderm formation during *Xenopus* embryogenesis.

**EXPERIMENTAL PROCEDURES**

*Xenopus Embryos and Microinjection—Eggs were obtained from *Xenopus laevis* primed with 600 units of human chorionic gonadotropin (DAE SUNG Microbiological Labs Co.), in vitro fertilized as described previously (12). Embryos were cultured in 0.33 × modified Ringer until stage 8 and then transferred to 0.1 × modified Ringer until they reached the appropriate stage for the experimentation outlined below. Developmental stages of the embryos were determined according to Nieuwkoop and Faber (13). Microinjection using a Nanoliter Injector (WPI) was used to inject cRNA or morpholino oligonucleotides into one-cell stage embryos.**
performed in 0.33× modified Ringer containing 4% Ficoll-PM400 (GE Healthcare).

**Plasmids, RNA Synthesis, and Morpholino Oligonucleotides—**
For expression in *Xenopus* embryos, the entire coding region of Xdpcp was cloned into the EcoRI and XbaI sites of the pCS2+ vector and GFP-pCS2+ vector, and into the Clal site of the Myc-pCS2+ vector. Deletion mutants of Xdpcp were cloned into the EcoRI and XbaI sites of the GFP-pCS2+ vector. Capped mRNAs were synthesized from linearized plasmids using the mMessage mMachine kit (Ambion). Xdcp, Xdpcp-Myc, and other deletion mutants of Xdpcp were linearized with NotI, and mRNA was synthesized using SP6 RNA polymerase. Antisense morpholino oligonucleotides (MO) were obtained from Gene Tools. The morpholino oligonucleotide sequences were as follows: *xdpcp* MO, 5'-TTCCAGAGTGAAA-GCCATCATGTTG-3'; control MO, 5'-CCTCCTTACCCT-CAGTTACAATTATA-3'. HA-hALK4, HA-hALK4(TD), HA-hALK4(KR), and Myc-Smad2 in the pCS2+ vector were linearized with NotI, and mRNA was synthesized using SP6 RNA polymerase.

In *Situ* Hybridization, RT-PCR, and Luciferase Reporter Assay—Whole mount *in situ* hybridization was performed with digoxigenin-labeled probes as described by Harland (14). Antisense *in situ* probes against *xdpcp* were generated by linearizing the pBSKII-*xdpcp* construct with XcmI and transcribing with the T7 RNA polymerase. RT-PCR analysis was performed as described (15). Primers and amplification cycles for RT-PCR analysis were as follows: *xdpcp* forward, 5'-GCCATCATGTTG-3'; *xdpcp* reverse, 5'-AGGATGGCAGGTACCTTTGTG-3' (25 cycles); primers for ODC, 5'-CCCAAAC-CCCAAGGAATGGT-3', and for *xdpcp* into the EcoRI and XbaI sites of the pCS2+ vector. Deletion mutants of Xdpcp were cloned into the EcoRI and XbaI sites of *Xenopus* embryos, the embryos were injected with the indicated reagents into the animal regions of the 4-cell stage, and animal cap explants isolated at stage 9 were separated into three pools.

**RESULTS**

**Cloning and Expression Patterns of Xdpcp—**Because PTB domain proteins play pivotal roles in many biological processes, we have attempted to identify the *in vivo* function of novel PTB domain proteins. Toward this goal, we cloned cDNA encoding a novel PTB domain protein using a PCR-based method and sequence information in the *Xenopus* EST data base (GenBank™ accession number BC073514) and named it *xdpcp* (*Xenopus* dok-PTB containing protein). *xdpcp* cDNA consists of 1782 nucleotides encoding a protein of 593 amino acids. PTB domains are divided into three groups, including Shc-like, IRS-like, and Dab-like (16). As implied by the name, Xdcp has a dok-PTB domain, one of the IRS-like PTB domains. To investigate the function of Xdcp in the *Xenopus* embryo, we first examined the spatial and temporal expression patterns of *xdpcp*, and found that it is strongly expressed in the animal hemisphere at the cleavage and blastula stages and around the anterior border of the neural plate and paraxial mesoderm at the tailbud stage. Whole mount *in situ* hybridization was performed at 4°C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 25 cycles. *An*, animal; *Vg*, vegetal; *D*, dorsal; *V*, ventral; *A*, anterior; *P*, posterior.

**Overexpression of Xdcp Leads to Defects in Mesendoderm Formation—**Based on its strong localization to the animal hemisphere at the late blastula stage, we focused on the function of Xdcp in early development. Thus, we addressed the effects of the gain-of-Xdcp function on the early patterning of
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FIGURE 2. Overexpression of xdpccp leads to defects in mesendoderm formation. A, xdpccp RNA (2 ng)-injected embryos show mesodermal defective phenotype. B, dorsal injection of xdpccp RNA inhibits the expression of endogenous mesodermal markers such as xbra, chordin, and goosecoid (gsc). Four-cell stage embryos were injected into the dorsal regions with 2 ng of xdpccp RNA or none, cultured until stage 10, and then subjected to in situ hybridization. C and D, RT-PCR analysis revealing that Xdpcp inhibits the expression of mesendodermal markers induced by activin in animal cap tissues, whereas it does not affect the expression of the 8mp target genes. +RT and −RT, control RT-PCR on the whole embryo RNA in the presence or absence of reverse transcriptase; AC, un.injected animal cap cells; (−), the animal cap cells injected with activin-BB or bmp4 RNA alone. Ornithine decarboxylase (ODC) serves as a loading control. E and F, luciferase assays in Xenopus embryos injected with combinations of indicated reagents. The amount of injected reagents is as follows: activin-BB RNA, 5 pg; Xnr-1 RNA, 100 pg; xdpccp RNA, 2 ng; ARE-Luc reporter DNA, 40 pg; 6DE-Luc reporter DNA, 40 pg. Error bars represent the standard deviation.

Xenopus embryos. Our analysis of embryos overexpressed xdpccp RNA in two dorsal blastomeres at the 4-cell stage revealed abnormal patterning such as bending and shortening of the anterior-posterior axis (Fig. 2A). We reasoned that this phenotype may be due to an abnormality in mesoderm formation. Therefore, we examined whether overexpression of xdpccp inhibits the expression of endogenous mesodermal markers such as xbra, chordin, and goosecoid. As shown in Fig. 2B, the expression of these mesodermal markers is remarkably reduced in embryos with xdpccp overexpression. These data suggest an inhibitory role of Xdpcp in mesoderm formation during Xenopus early development.

As mentioned previously, PTB domain proteins are also related to the activin/nodal signaling pathway, which is involved in mesendoderm formation during Xenopus early development. Therefore, we initially hypothesized that Xdpcp regulates mesendoderm formation by inhibiting activin/nodal signaling. To address whether overexpression of xdpccp affects mesendoderm formation and activin/nodal signaling, we performed RT-PCR analysis. Functional analysis in Xenopus animal caps showed that the injection of activin RNA could induce mesodermal markers such as xbra, chordin, and mix2 as well as endodermal markers such as sox17, endodermin, and vegT, and the induction of these markers was inhibited by coexpression of xdpccp RNA (Fig. 2C). However, Xdpcp did not affect the expression of BMP-induced genes such as msx-1 and xvent-1, suggesting that Xdpcp has a specific function in activin/nodal signaling (Fig. 2D). To examine the effects of Xdpcp on activin/nodal signaling-dependent promoter activity, we carried out various luciferase assays using an ARE-Luc reporter containing three copies of an ARE (activin-response element of the Xenopus mix2 promoter) (17) or a 6DE-Luc reporter containing six copies of a DE (activin/nodal-inducible distal element of the Xenopus gsc promoter) (18). Similar to our findings with RT-PCR analysis, we observed that Xdpcp diminished transcriptional activity associated with activin/nodal signaling (Fig. 2, E and F). These results suggest that Xdpcp inhibits the formation of mesendoderm by activin/nodal signaling.

Xdpcp Knockdown Leads to Expansion of Mesodermal Territory—To investigate whether Xdpcp is indispensable for regulating mesendoderm formation, we carried out loss-of-function analysis using antisense MO capable of depleting the Xdpcp protein (19). We designed a morpholino to target the start codon region of the xdpccp gene to disrupt translation of the xdpccp mRNA. To confirm the efficiency and targeting specificity of the xdpccp MO, we coinjected the MO with Myc-tagged xdpccp RNAs with and without the MO targeting sites (untranslated region and open reading frame, respectively), and performed Western blot analysis with anti-Myc antibody. As shown in Fig. 3A, xdpccp MO can specifically block production of the Xdpcp protein. To examine the effects of Xdpcp depletion on embryonic patterning and mesoderm formation in Xenopus embryos, we injected xdpccp MO into the dorsal-animal regions of 4-cell stage embryos and then observed its effects on the patterning of embryos and the expression of endogenous mesodermal markers such as xbra and goosecoid. Intriguingly, xdpccp knockdown embryos revealed a slightly dorso-anteriorized phenotype, which is translated into the dorso-anterior index 6 (20) (Fig. 3B). This demonstrates that activin/nodal signaling might be increased in response to Xdpcp depletion, because activin/nodal activity induces dorso-anterior fates. Xdpcp-depleted embryos also show an expansion in the expression of mesodermal markers toward the ectodermal territory (Fig. 3C). To obtain more evidence that Xdpcp inhibits mesendoderm formation of Xenopus embryos, we performed RT-PCR analysis using mesodermal markers such as xbra, chordin, and mix2 and endodermal markers such as sox17 and endodermin. As shown in Fig. 3D, endogenous expression of these markers was decreased by xdpccp overexpression, whereas xdpccp knockdown leads to the increase of these expression. These data suggest that the presence of Xdpcp in the ectodermal region prevents the mesendodermal territory from expanding.
Injection of activin-βB induced phosphorylation of Smad2, and this phosphorylation was reduced by coexpression of xdpcp. Interestingly, Xdpcp depletion by MO induced more phosphorylation of Smad2, which demonstrates that the endogenous function of Xdpcp is to negatively regulate activin/nodal signaling. However, Xdpcp did not affect Smad1 phosphorylation induced by Bmp signaling (Fig. 4D).

Because Xdpcp inhibits the phosphorylation of Smad2, we examined whether it also affects the next step in the pathway, the nuclear translocation of Smad2. The results of immunostaining analysis show that activin-induced nuclear accumulation of Smad2 is inhibited by coexpression of xdpcp (Fig. 4E). Because Xdpcp inhibits the phosphorylation and nuclear translocation of Smad2, we tested whether Xdpcp is incapable of inhibiting the activin/nodal signaling induced by constitutively active Smad2 (CA-Smad2) (21). As shown in Fig. 5A, the results of RT-PCR analysis revealed that CA-Smad2 could induce the expression of mesendodermal markers, and this induction was unaffected by xdpcp overexpression. Next, we examined whether the morphological defects induced by xdpcp overexpression could be rescued by coexpression of CA-Smad2. xdpcp overexpression caused abnormal embryonic patterning such as the shortening and bending of the anterior-posterior body axis, and these phenotypes were partially rescued by coexpression of CA-Smad2 (Fig. 5, B and C). Together, these results indicate that Xdpcp acts upstream of Smad2 in the activin/nodal signaling pathway.

Xdpcp Inhibits the Interaction between Activin Receptor and Smad2—Based on our finding that Xdpcp functions upstream of Smad2 in the pathway, we asked whether Xdpcp can interact with Alk4, a type 1 receptor, or Smad2. To answer this, we injected activin-βB, xdpcp RNA, and xdpcp MO into the animal region of 4-cell stage embryos. We then isolated animal caps at the blastula stages, cultured them to stage 10, and then performed a Western blot analysis with phospho-Smad2 antibody (Fig. 4C).
To address the inhibitory mechanism of Xdpcp in activin/nodal signaling, we examined whether the interaction between activin receptor and Smad2 under activin signaling is affected by coexpression of Xdpcp. Thus, we injected RNAs as indicated in Fig. 6 into the animal regions of 2-cell stage embryos, cultured the injected embryos until stage 10, and then performed an immunoprecipitation assay. Intriguingly, Smad2 interacts with Alk4 under activin signaling and this interaction is inhibited by coexpression of Xdpcp (Fig. 6C).

Because Xdpcp inhibits the association of Smad2 with Alk4, we asked whether the same interface of Alk4 is involved in binding to Xdpcp and Smad2. To test this, we cloned a mutant form of Alk4, which is deleted in the L45 region known as the Smad-binding site, then examined the capability of the mutant Alk4 to associate with Xdpcp and Smad2. As shown in Fig. 6D, wild type Alk4 interacts with Xdpcp and Smad2, whereas the L45-deletion mutant of Alk4 did not bind to both Xdpcp and Smad2. This indicates that Xdpcp and Smad2 competitively interact with the same region of Alk4. To better understand the mechanisms for Xdpcp action, we examined subcellular localization of Xdpcp in both cultured cells and Xenopus embryonic cells. Consistently, the results of immunostaining analysis reveal that Xdpcp is localized in the cytoplasm, but not nucleus of HeLa cells (Fig. 7A), and it is colocalized with Alk4 in the cytoplasmic vesicles and plasma membrane of both HeLa cells and Xenopus animal cap cells (Fig. 7B). These subcellular localization data support the action mechanism of Xdpcp that interacts with Alk4 and inhibits the association of Alk4 with Smad2 in the cytoplasmic vesicles and plasma membrane. Taken together, these results suggest that Xdpcp may inhibit activin/nodal signaling by masking the receptor, thereby inhibiting the interaction between activin receptor and Smad2.

To test our hypothesis that Xdpcp inhibits the activin/nodal signaling by masking the activin receptor, we examined whether interaction between Xdpcp and the receptor is essential for the inhibitory roles of Xdpcp in activin/nodal signaling. To test this, we designed several deletion mutants of Xdpcp (Fig. 8A), transfected them into HEK293FT cells, and then carried out an immunoprecipitation assay. As shown in Fig. 8B, the PTB-deletion mutant (Xdpcp-ΔN2) cannot bind to Alk4, whereas wild type (Xdpcp-fl) and PTB-including mutants (Xdpcp-ΔN1 and -ΔC) interact with the activin receptor. This indicates that the PTB domain is essential for interaction between Alk4 and Xdpcp. Next, to test whether the PTB-deletion mutant lacks the inhibitory function in activin/nodal signaling, we analyzed the effects of PTB deletion on the transcriptional activity of luciferase reporters driven by the goosecoid promoter responding to the activin/nodal signals. As shown in...
Fig. 8C, the transcriptional response stimulated by Xnr-1 (Xenopus nodal-related-1) is down-regulated by coexpression of xdpcp. However, coexpression of the PTB-deletion mutant (Xdpcp-ΔN2), which is incapable of interacting with Alk4, did not affect that response and coexpression of the PTB-containing mutants (Xdpcp-ΔN1 and -ΔC), which are capable of interacting with the receptor, resulted in down-regulation of Xnr-1. These data suggest that interaction of Xdpcp with Alk4 via its PTB domain is essential for negative regulation of activin/nodal signaling. Taken together, the results suggest a model in which Xdpcp inhibits activin/nodal signaling by masking the receptor, thereby protecting the ectodermal fate from activin/nodal signals capable of inducing mesoderm during early embryogenesis (Fig. 9).

**DISCUSSION**

In this study, we have provided evidence that a novel PTB protein, Xdpcp, negatively regulates the activin/nodal signaling pathway in germ-layer specification during Xenopus development. First, xdpcp is expressed in the prospective ectodermal region in the Xenopus early embryo. Second, gain-of- and loss-of-Xdpcp function lead to abnormal mesoderm formation and embryonic patterning in the Xenopus embryo. Third, using RT-PCR, luciferase assay, and axis duplication assay, we found that Xdpcp inhibits responses to the activin/nodal signaling. In addition, Xdpcp inhibits phosphorylation and nuclear translocation of Smad2. Finally, Xdpcp binds to Alk4, a type I receptor and interferes with the interaction between Alk4 and Smad2. Together, these results indicate that the Xdpcp-Alk4 interaction mediates inhibition of Smad2 binding and phosphorylation, thus regulating activin/nodal signaling. This Xdpcp-mediated regulation of the activin/nodal signaling pathway is pivotal for correct germ-layer specification in Xenopus embryogenesis.

**Germ-layer Specification and Activin/Nodal Signaling**—Regulation of germ-layer specification is one of the most critical and classical problems in vertebrate development. As mentioned previously, mesoderm is formed by mesoderm-inducing signals...
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FIGURE 7. The subcellular localization of Xdpccp. A, Xdpccp is localized at the cytoplasm, but not nucleus in HeLa cells. HeLa cells were transfected with GFP-tagged Xdpccp, treated with or without activin protein (50 ng/ml) for 10 min after 36 h of transfection. B, Xdpccp is co-localized with Alk4 in both HeLa cells (top) and Xenopus animal cap cells (bottom). Scale bars are 20 (HeLa cells) and 50 μm (animal cap cells).

such as activin/nodal signaling from the underlying endoderm in the Xenopus embryo. In addition, mesoderm-restriction factors from the overlying ectoderm are also required for determining the proper territory of the mesoderm. Recently, several maternal proteins including Coco (22), Ectodermin (23), Sox3 (24), and serum-response factor (SRF) (25) and zygotic proteins including Xema (26) and XFDL156 (27) have been identified as mesoderm-restriction factors in Xenopus germ-layer specification and most of these factors are involved in regulating the activin/nodal signaling pathway. Briefly, Coco is a secreted antagonist of BMP, TGF-β, and Wnt ligands, and Sox3 inhibits nodal expression and induces other regulators such as Ectodermin, Xema, and Coco. In addition, Ectodermin controls the level of Smad4 as an ubiquitin ligase, and serum response factor inhibits the formation of the Smad2-Fast-1 complex. Finally, XFDL156 inhibits the function of p53 cooperating with Smad2. These findings provide evidence that the tight regulation of activin/nodal signaling at multiple points is involved in correct germ-layer formation. Consistent with this concept, we have shown that Xdpccp is involved in the proper formation of embryonic germ-layers by inhibiting activin/nodal signaling through disruption of the association between Alk4 and Smad2.

Negative Regulation of Activin/Nodal Signaling—TGF-β family growth factors including activin and nodal-related proteins are pivotal for controlling diverse cellular processes such as cell proliferation, apoptosis, migration, and differentiation and disturbances of these signals can cause many serious human diseases and disorders. Therefore, it is very important to clarify the fine molecular mechanisms regulating these signals. Many molecules have been identified as negative regulators to achieve tight regulation of the TGF-β signaling pathway (28). Especially when it comes to the regulation at the level of type I receptor, which has three regulatory points including dephosphorylation, degradation of type I receptor, and interference of R-Smad binding and phosphorylation. The inhibitory Smads (Smad6 and -7), known as core players in the negative regulation of TGF-β signaling, are involved in regulation of receptors, including the competitive inhibition of R-Smad binding (29), degradation of active receptors by interacting with Smurfs (30), dephosphorylation of active receptors (31), and inhibition of Smad-dependent promoter activation (32). In addition, phosphatases such as PP1c (31) and PP1α (33) dephosphorylate the type I receptor and HECT domain E3-ubiquitin ligases such as Smurf1/2 (30, 34), NEDD4–2 (35), and Tiul1/WWP1 (36, 37) are involved in degradation of the type I receptor. Moreover, Akt has been suggested to bind to Smad3, thereby sequestering it from the type I receptor in an Akt kinase-independent manner (38, 39). However, there is still controversy over whether Akt inhibits Smad3 phosphorylation via mTOR in an Akt kinase-dependent manner (40). We suggest that Xdpccp inhibits activin/nodal signaling by interacting with Alk4, a type I receptor, and masking it from Smad2. Xdpccp is a novel regulator that competes with Smad2 for binding to Alk4. Like Xdpccp, Smad7 was originally reported as a negative regulator inhibiting the association between type I receptor and R-Smad. In addition, Smad7 is also involved in the dephosphorylation and degradation of the type I receptor by protein phosphatases and ubiquitin ligases. However, we did not observe an association between Xdpccp and Smad7 (data not shown), and the relationship of Xdpccp with other molecules implicated in the regulation of type I receptor and other mechanisms of Xdpccp function remains to be further elucidated.

The Role of PTB Protein in Activin/Nodal Signaling—Here we report on a novel PTB protein, Xdpccp, that is implicated in activin/nodal signaling. Growth factor receptors generally employ adaptor proteins for stabilization and amplification of signaling (41). PTB domains, which include IRS-like, Shc-like, and Dab-like domains, are found in many adaptor proteins (16). In particular, in the activin/nodal pathway of TGF-β signaling, two PTB adaptor proteins, Dab2 and Dok1, play similar roles promoting the signals between receptors and R-Smad (7, 8). Dok1 has an IRS-like PTB domain similar to Xdpccp, whereas Dab2 has a Dab-like PTB. Regardless of the similarity between PTB domains, our data show that Xdpccp possesses an opposite role to that of Dok1 in activin/nodal signaling. Unlike Dok1, Xdpccp lacks a PH domain and does not interact with Smad2 or -4 proteins that bind to the N-terminal PH and PTB domains of Dok1. This finding suggests that there are structural differences in the PTB region between Dok1 and Xdpccp. Moreover, our data using deletion mutants show that the association of Alk4 with the PTB domain of Xdpccp is essential for negative regulation of activin/nodal signaling (Fig. 8). In addition, Yamakawa and colleagues (8) reported that a deletion mutant (Dok PP) of Dok1 composed of only PH and PTB domains down-regulates activin A- and TGF-β-induced promoter activity, indicating
that another region of Dok1 is related to up-regulation of the signaling. We reason that these differences between Dok1 and Xdpcp lead to their opposite roles in activin signaling.

In summary, our study reveals that Xdpcp plays a crucial role in correct germ-layer formation during vertebrate embryogenesis by disrupting the interaction between Alk4 and Smad2, resulting in the negative regulation of activin/nodal signaling. The detailed mechanisms by which Xdpcp regulates activin/nodal signaling in embryo development remain to be further elucidated.

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