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Integrin-α3 mediates binding of Chordin to the cell surface and promotes its endocytosis

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Dorsoventral patterning in animal development is regulated by a morphogenetic gradient of Bone morphogenetic protein signalling, which is established by a set of proteins that are conserved from *Drosophila* to vertebrates. These include Chordin (Chd)/Short gastrulation, Xolloid/Tolloid and Twisted gastrulation. Here, we report the identification of a cell-surface component of this morphogenetic pathway. Prompted by the observation that Chd protein bound to the surface of certain cell lines with subnanomolar affinity, we identified two cell-surface proteins that bind to Chd, one of which corresponds to Integrin-α3. Integrin-α3 and Chd are co-expressed in the *Xenopus* embryo. Transfection of Integrin-α3 increased the binding of Chd to the cell surface, which was competed by an excess of soluble Integrin-α3. After binding to the cell surface, Chd was translocated into intracellular endocytic compartments in a temperature-dependent manner. We propose that Integrin-α3 may regulate the concentration of Chd protein in the extracellular space by endocytosis.

INTRODUCTION

Embryonic patterning arises through the generation of morphogen gradients during early development. Dorsoventral patterning of fly and vertebrate embryos has been well studied, and is one of the paradigms for understanding morphogen function (Freeman & Gurdon, 2002). The establishment of an extracellular gradient of Bone morphogenetic protein (BMP) determines cell-fate decisions along the dorsoventral axis. A gradient of this morphogen is generated in the extracellular space by the action of a set of evolutionarily conserved secreted proteins, including Chordin (Chd)/Short gastrulation (Sog), Xolloid (Xld)/Tolloid (Tld) and Twisted gastrulation (Tsg; De Robertis *et al.*, 2000). The

Drosophila Chd homologue, Sog, is a key component in the generation of embryonic BMP/Decapentaplegic (Dpp) gradients (Eldar *et al.*, 2002). Sog can diffuse along the dorsoventral axis, and its diffusion and extracellular concentration levels are enhanced by a *dynamin* mutation that blocks endocytosis (Srinivasan *et al.*, 2002). Chd and Sog encode secreted proteins of ~120 kDa that contain four cysteine-rich repeats (CRs). The CR modules bind directly to BMP, preventing its binding to the BMP receptor (Piccolo *et al.*, 1996; Larraín *et al.*, 2000). The anti-BMP activity of Chd is controlled by two cofactors, Tld and Tsg. Tld is a zinc metalloproteinase that inactivates Chd by proteolytic cleavage at sites located just after CR1 and CR3 (Piccolo *et al.*, 1997; Scott *et al.*, 1999). Tsg is an evolutionarily conserved secreted protein that forms ternary complexes with BMP4 and Chordin that are unable to signal in the absence of Tld. Tsg binding also makes Chd a better substrate for cleavage by Tld, and competes the anti-BMP activity of Chd proteolytic fragments. Thus, Tsg promotes BMP activity in the presence of Tld (Oelgeschläger *et al.*, 2000; Ross *et al.*, 2001; Larraín *et al.*, 2001).

This work was initiated in an attempt to identify other components of the Chd/BMP/Tsg/Tld regulatory pathway. It was found that Chd binds to the cell surface of various tissue-culture cell lines. To identify the molecular basis of this interaction, we used affinity-chromatography Chd columns and found that two cell membrane proteins bound to Chd. One of them was identified as the transmembrane receptor Integrin-α3. An excess of soluble purified Integrin-α3 prevented the binding of Chd to the cell surface. The cell-surface interaction between Chd and Integrin-α3 was followed by endocytosis at 37 °C. Chd and Integrin-α3 are coordinately expressed in the dorsal mesoderm of *Xenopus* embryos. We propose that the levels of Chd in the extracellular space may be regulated by endocytosis through Integrin-α3.

RESULTS AND DISCUSSION

During screening for novel Chd-interacting proteins using a secretion-trap expression cloning method (Davis *et al.*, 1996), we noticed that non-transfected COS-7 cells bound detectable amounts of a Chd–alkaline-phosphatase fusion protein (Chd–AP). When binding was quantified using a biochemical fluorescent AP assay, Chd–AP bound to COS-7 cells in amounts significantly higher than background levels (Fig. 1A). As negative controls, we used a secreted version of AP (s-AP) and a Tsg–AP fusion protein,

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neither of which bound to COS-7 cells in detectable amounts (Fig. 1A; and data not shown). We were able to saturate the binding of Chd–AP to COS-7 cells at 4 °C using increasing concentrations of Chd–AP protein, with an estimated dissociation constant (K_d) of 0.24 nM (Fig. 1B). This binding was specific, as it was competed by excess purified mouse Chd protein (Fig. 1C).

Chd–AP binding was lost from COS-7 cells that were pretreated with 0.1% or 1% Triton X-100 (Fig. 1D); these amounts of detergent are known to solubilize integral plasma membrane proteins, but not extracellular matrix (ECM) components (Mehta *et al.*, 1985). This suggested that Chd binds to a cell membrane protein. To visualize binding to the cell surface, 10T1/2 fibroblasts were incubated at 4 °C with a protein consisting of Chd fused to the Fc
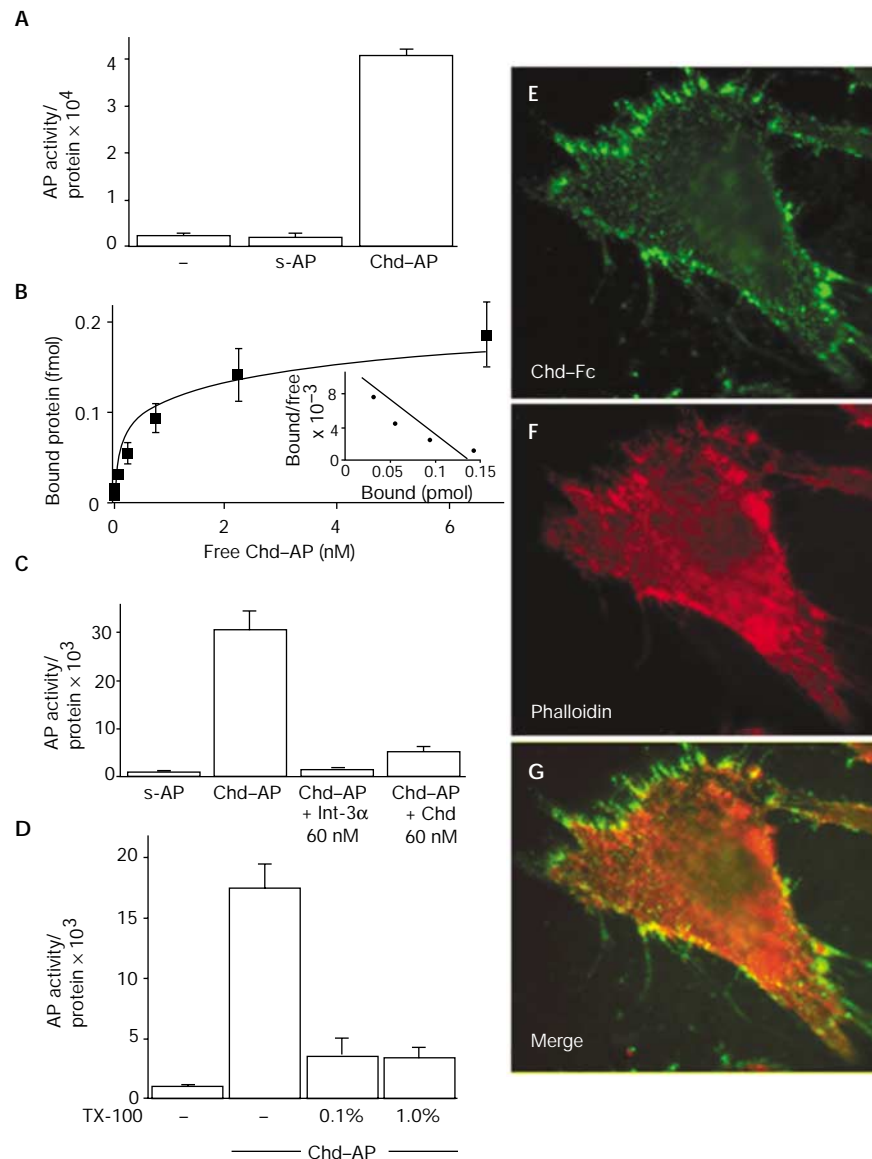

Fig. 1 | Chordin binds to the cell surface. (**A**) Chordin–alkaline-phosphatase (Chd–AP) binds to COS-7 cells. COS-7 cells were incubated with Chd–AP or secreted AP (s-AP) for 2 h at 4 °C, and the amount of activity of bound AP was quantified using a fluorescent AP substrate. Equal loading was confirmed by protein measurements. (**B**) Binding curve and Scatchard analysis of Chd–AP binding to COS-7 cells. A dissociation constant (K_d) of 0.24 nM was estimated. (**C**) COS-7 cells were incubated with Chd–AP in the presence of 60 nM recombinant Integrin-α3 (Int-α3; Chemicon) or mouse Chd (R&D Systems), and the amount of bound Chd–AP was quantified using a fluorescent AP substrate. Other cysteine-rich-repeat-containing proteins were not tested because they are not available in purified form. (**D**) Chd binding requires an intact cell membrane. COS-7 cells were incubated with 0.1% or 1.0% Triton X-100 (TX-100), and Chd–AP was bound and quantified. (**E**) Chd binding colocalizes with actin filaments. 10T1/2 fibroblasts were incubated with pure Chd–Fc at 4 °C, fixed and permeabilized, and binding was visualized with an anti-human IgG antibody conjugated to Alexa Fluor 486. (**F**) The same cell as in (**E**), stained with phalloidin-Alexa558 to visualize actin. (**G**) Merge of (**E**) and (**F**). Note the overlap between Chd–Fc and actin (yellow).

fragment of human IgG (Chd–Fc), fixed, and stained with a fluorescent anti-human IgG. Chd–Fc binding was detected in membrane ruffles, filopodia and structures reminiscent of focal-adhesion plaques, particularly at the leading edge of the cell (Fig. 1E). Chd–Fc co-localized with actin filaments stained by fluorescent phalloidin, indicating that some Chd binding sites overlap with focal-adhesion plaques (Fig. 1F,G, note yellow staining where the Chd and actin signals overlap). These results show that Chd can bind with high affinity (K_d in the subnanomolar range) to the cell surface in cultured cell lines.

To identify the cell-surface proteins that bind to Chd, we prepared a Chd affinity-chromatography column. Chd–Fc and secreted Fc (s-Fc) fusion proteins were produced in S2 cells, purified with protein A, and covalently crosslinked to protein-A–agarose beads

using dimethylpimelimidate (DMP). Cell-surface proteins from COS-7 cells were biotinylated (using EZ-link-Biotin; Pierce), solubilized with 0.1% Triton X-100 (as in Fig. 1D), and loaded on Chd–Fc or s-Fc affinity columns. Bound proteins were eluted with SDS and visualized by performing a streptavidin–horseradish-peroxidase (SA–HRP) immunoblot. As shown in Fig. 2A, lane 3, the Chd–Fc column bound two proteins that migrated in non-reducing SDS gels with apparent molecular weights of 170 and 200 kDa. Neither protein bound to the s-Fc control column (Fig. 2A, lane 2). The molecular weight of these proteins and the localization of the Chd–Fc staining on focal-adhesion plaques (Fig. 1E–G) suggested integrins as candidate cell-surface receptors for Chd.

Integrins constitute a large transmembrane receptor family of at least 24 distinct heterodimers, and were originally described as
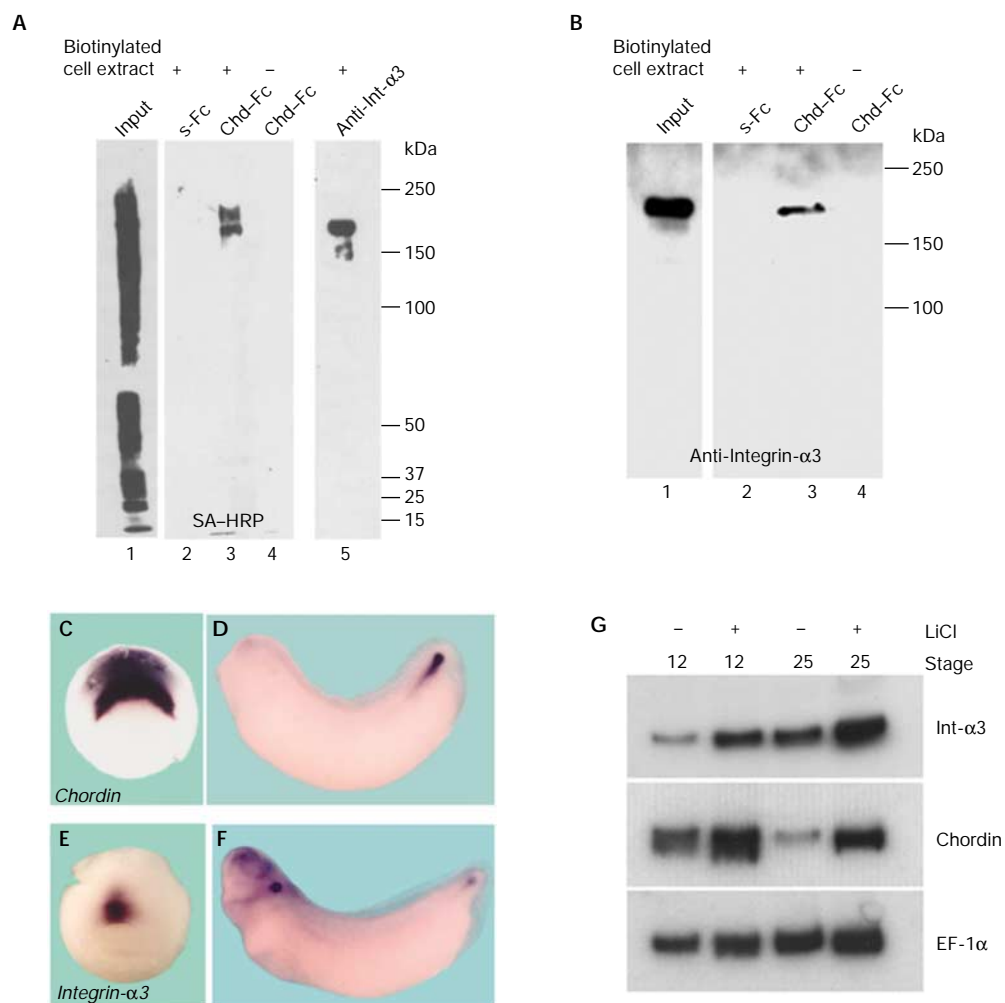

Fig. 2 | Chordin affinity columns bind biotinylated cell-surface Integrin-α3. (**A**) A Chordin (Chd)–Fc affinity matrix binds two distinct cell-surface proteins in COS-7 cells. Cell extracts containing biotinylated surface proteins from COS-7 cells were bound to secreted Fc (s-Fc; lane 2) or Chd–Fc (lane 3) columns, or were immunoprecipitated using an anti-Integrin-α3 (Int-α3) antibody (lane 5). Proteins bound to the columns were analysed by immunoblotting with streptavidin–horseradish-peroxidase (SA–HRP; Pierce). Lane 1 shows loading of 1% of the total biotinylated cell lysate. (**B**) A Chordin affinity matrix binds Integrin-α3. COS-7 cell extracts were bound to s-Fc (lane 2) or Chd–Fc affinity columns (lane 3), eluted, and analysed by immunoblotting with anti-Integrin-α3. (**C**–**G**) Chordin and Integrin-α3 are co-expressed during embryonic development. *In situ* hybridization analysis of *chordin* (**C**,**D**) and *integrin-a3* (**E**,**F**) expression at stages 10.5 and 32. (**G**) RT–PCR (PCR after reverse transcription) analysis of *chordin* and *integrin-a3* messenger RNA levels in control and LiCl-treated embryos. Elongation factor-1α (EF-1α) was used as a loading control.
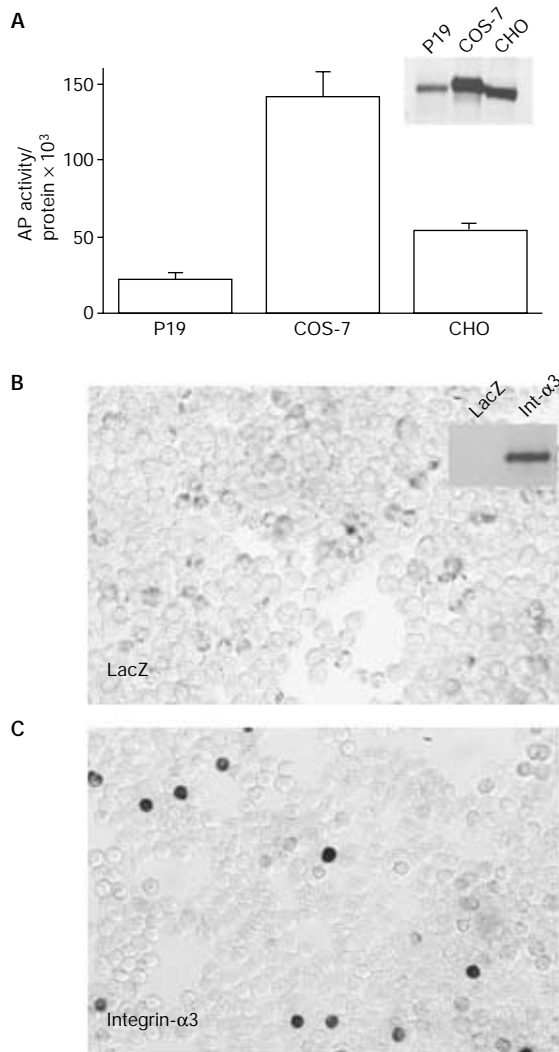
A

B

LacZ

C

Integrin-α3

Fig. 3 | Chordin binds to the cell surface through Integrin-α3. (**A**) Binding of Chordin–alkaline-phosphatase (Chd–AP) to P19, COS-7 and CHO cell lines. The inset shows an anti-Integrin-α3 immunoblot of cell extracts from the three cell lines. The amount of Chd–AP binding correlates with endogenous levels of Integrin-α3. (**B,C**) Chd–AP binds to 293T cells transfected with *integrin-a3*. 293T cells were transfected with *LacZ* or human *integrin-a3* full-length complementary DNAs, incubated with recombinant Chd–AP and stained for AP activity. The inset shows an anti-Integrin-α3 (Int-α3) immunoblot of the corresponding cell extracts.

one of the main components of focal-adhesion plaques in fibroblasts (Hynes, 2002). Among them, Integrin-α3 stood out as an attractive candidate because it is co-expressed with Chd in the dorsal blastopore lip and its derivatives (the notochord and chordoneural hinge) and is coordinately upregulated with Chd in embryos that are dorsalized by lithium chloride treatment (Fig. 2C–G; Sasai *et al.*, 1994; Whittaker & DeSimone, 1991; Meng *et al.*, 1997). As Chd and Integrin-α3 are coordinately regulated in the dorsal mesoderm, it seemed possible that they might function in the same biochemical pathway. Furthermore, Integrin-α3 migrates with an apparent molecular weight of 170 kDa in

non-reducing SDS gels (Shang *et al.*, 2001). For these reasons, we tested whether Integrin-α3 is one of the Chd cell-surface receptors that is expressed in COS-7 cells. An anti-Integrin-α3 antibody immunoprecipitated a biotinylated protein with a molecular weight similar to the smaller band bound by the Chd–Fc column (Fig. 2A, compare lanes 3 and 5). Furthermore, the 170-kDa protein purified by the Chd–Fc affinity matrix was specifically recognized by the anti-Integrin-α3 antibody in immunoblots (Fig. 2B, lane 3). Thus, one of the cell-surface proteins that interact with Chd corresponds to Integrin-α3. The nature of the 200-kDa protein remains unknown; one possibility is that it corresponds to Integrin-β1, the heterodimerization partner of Integrin-α3, but this seems unlikely as the expected molecular weight of Integrin-β1 in non-reducing gels is 110 kDa (Shang *et al.*, 2001).

We next tested whether the binding of Chd to COS-7 cells could be mediated by Integrin-α3. First, an excess of recombinant purified Integrin-α3 blocked the binding of Chd–AP to COS-7 cells (Fig. 1C). Second, we found that the amount of Chd–AP bound to the different cell lines correlated with the endogenous levels of Integrin-α3 expression (Fig. 3A). P19 cells, which express low levels of Integrin-α3 bound approximately six times less Chd–AP than did COS-7 cells, which express higher levels of Integrin-α3 protein (Fig. 3A, inset). 293T cells, which do not express detectable levels of Integrin-α3, did not bind Chd–AP (Fig. 3B). Third, to test whether Integrin-α3 was sufficient for binding of Chd to the cell surface, 293T cells were transfected with a full-length human integrin-α3 complementary DNA and stained with Chd–AP. We found that 293T cells transfected with integrin-α3 bound Chd–AP to the cell surface (Fig. 3C). We conclude from these experiments that Integrin-α3 mediates the binding of Chd to the cell surface.

Recent studies have highlighted the importance of receptor-mediated endocytosis in the formation of morphogen gradients in the embryo (Lander *et al.*, 2002; Seto *et al.*, 2002; Vincent & Dubois, 2002). For example, BMP/Dpp diffusion can be regulated by endocytosis after binding to its signalling receptor in imaginal discs (Entchev *et al.*, 2000), and the secreted protein Dickkopf regulates Wingless/Int-related (Wnt) signalling by inducing the rapid removal of the Wnt co-receptor lipoprotein-receptor-related protein 6 (LRP6) by endocytosis, together with the transmembrane protein Kremen (Mao *et al.*, 2002). Integrins are transmembrane glycoproteins that link the ECM to intracellular cytoskeletal and signalling networks, which, in concert, regulate cell adhesion, migration, cell–cell communication, proliferation and survival (Kreidberg, 2000). One of the functions described for integrins is their ability to mediate the internalization of ECM components, adenoviruses and apoptotic cells through endocytosis (Memmo & McKeown-Longo, 1998; Li *et al.*, 2001; Hanayama *et al.*, 2002).

To determine whether Integrin-α3 regulates extracellular levels of Chd through endocytosis, we analysed the cellular localization of Chd–Fc in COS-7 cells using a fluorescent probe that marks endocytic vesicles (Entchev *et al.*, 2000; Texas Red Dextran; molecular weight 3,000 Da). When incubated at 4 °C, the Chd–Fc signal was detected on the cell surface (Fig. 4A–D). To allow internalization, cells were shifted to 37 °C for 30 min. After this treatment, Chd–Fc colocalized with Texas Red Dextran in punctate intracellular vesicles (Fig. 4E–H; note yellow spots in the inset in Fig. 4G). When COS-7 cells were transfected with full-length *integrin-a3* cDNA and stained with Chd–AP, the amount of intracellular Chd–AP was greatly increased compared with
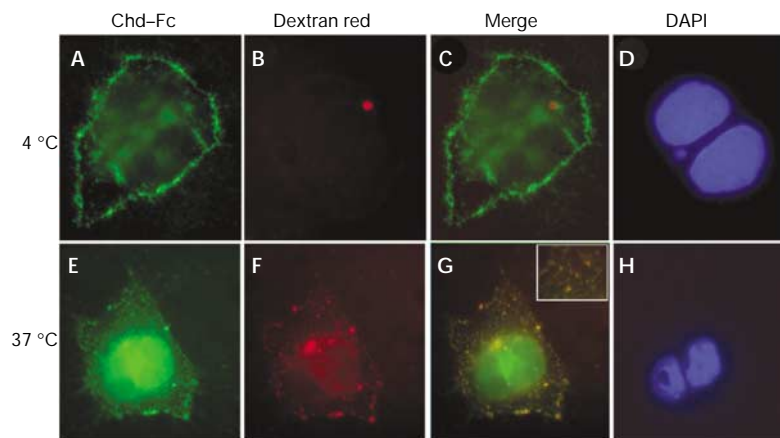
Fig. 4 | Chordin–Fc translocates into intracellular endocytic compartments at 37 °C. Cells were incubated on ice with Chordin (Chd)–Fc for 2 h, washed, and then incubated with Texas-Red–dextran for 30 min on ice (**A–D**) or at 37 °C (**E–H**). (**A,E**) Localization of Chd–Fc (green) bound to COS-7 cells. (**B,F**) Localization of Texas-Red–dextran (red) in punctate endocytic compartments after shifting to 37 °C. (**C,G**) Merged images. (**D,H**) 4,6-diamidino-2-phenylindole (DAPI) staining of nuclear DNA. Note that at 4 °C, most of the Chd–Fc is detected at the cell surface, but after 30 min at 37 °C it colocalizes with endocytic vesicles in the cytoplasm that contain Texas-Red–dextran (**G**). The red spot in (**B**) is artefactual, but shows that red fluorescence does not bleed into the green fluorescence channel.

untransfected COS-7 cells (data not shown). These results indicate that binding of Chd to Integrin-α3 on the surface of COS-7 cells results in its endocytosis and removal from the extracellular space in a temperature-dependent manner.

Recently, a gradient of the Sog protein has been directly visualized in the *Drosophila* embryo and modified using the temperature-sensitive *shibire* (*shi*ts) endocytosis mutant (Srinivasan *et al.*, 2002). Interestingly, at the nonpermissive temperature, blockage of endocytosis increases the extracellular levels of Sog and enhances its diffusion towards the dorsal pole of the fly embryo (Srinivasan *et al.*, 2002). Thus, the Sog gradient is regulated and maintained by endocytosis. Chd and Integrin-α3 are co-expressed during *Xenopus* development. Integrin-α3 may limit Chd diffusion by endocytosis and removal from the extracellular space or, alternatively, may facilitate transcytosis, regulating the shape of a hypothetical Chd gradient. The experiments presented here show that Chd can bind to the cell surface through Integrin-α3 in cell culture, and that this binding can result in the removal of Chd from the extracellular space. Previous work has shown that endocytosis constitutes an important step in the formation of morphogenetic gradients (Entchev *et al.*, 2000; Lander *et al.*, 2002; Seto *et al.*, 2002; Vincent & Dubois, 2002). A possible functional interaction between Chd and Integrin-α3 during *Xenopus* embryonic development is independently supported by recent observations in *Drosophila* (Araujo *et al.*, 2003). Genetic interactions have been detected between *sog* and several integrin-coding genes (*myospheroid*, *multiple edematous wing* and *scab*) during the formation of wing veins in pupal development. In addition, in co-immunoprecipitation experiments, it was found that α-PS1 integrin, which is encoded by *multiple edematous wing*, binds to the Sog protein (Araujo *et al.*, 2003). Interestingly, *Drosophila* α-PS1 integrin is most similar to vertebrate integrin-α3. We propose that the interaction between Integrin-α3 and Chordin provides another evolutionarily conserved mechanism for modulating BMP/Dpp signalling in the extracellular space.

METHODS

Affinity chromatography and biotinylation. Chd–Fc and s-Fc fusion proteins were produced in stable *Drosophila* S2 cell lines. For purification of Fc fusion proteins, 1 l of *Drosophila* Serum-free Medium (Invitrogen) containing the secreted proteins was bound to 1 ml of protein-A–sepharose, and the column was washed with 50 ml of 0.01 M sodium phosphate, pH 8.0, 150 mM NaCl. Proteins bound to protein A were eluted with five column-volumes of 0.1 M glycine, pH 3.0, and fractions of 1 ml were collected and neutralized in tubes containing 50 μl of 1 M Tris-HCl, pH 9.0. The proteins purified in this way were bound to protein-A–agarose beads (~0.5 mg protein per ml of wet beads) and crosslinked using DMP (Pierce; Harlow & Lane, 1988). For biotinylation, COS-7 cells were grown to confluency, washed twice with ice-cold PBS, and incubated on ice for 30 min with 1.0 mg ml^{-1} EZ-link sulpho-NHS-biotin (Pierce) in PBS. The reaction was stopped by washing cells twice with ice-cold PBS and incubating them for 30 min with 0.1 M glycine in PBS. Two 100-mm plates of cells were lysed with lysis buffer (10 mM Tris pH 7.5, 0.15 M NaCl, 10% glycerol, 0.1% Triton X-100; with complete protease inhibitor cocktail, EDTA-free (Roche Biochemicals)). Chd–Fc and s-Fc beads were incubated for 16 h at 4 °C with cell lysates in lysis buffer supplemented with 2 mM of each of the following divalent cations: Mg^{2+}, Ca^{2+} and Mn^{2+}. Beads were washed four times with ice-cold lysis buffer, and bound proteins were eluted with SDS loading buffer and analysed by immunoblotting using SA–HRP (Pierce) at a dilution of 1 in 4,000.

Cell-surface binding. Cells were grown to 80% confluence in 24-well plates, washed twice with 1 ml of ice-cold HBSS containing 0.5 mg ml^{-1} BSA and 20 mM Hepes, pH 7.0, and incubated for 2 h on ice with 250 μl of AP fusion protein (Flanagan & Cheng, 2000). Cells were then washed on ice six times with 1 ml of ice-cold HBSS and extracted with 100 μl of 10 mM Tris-HCl, pH 7.5, containing 1% Triton X-100. Endogenous AP activity was inactivated by incubation of the plate at 65 °C for 1 h. AP activity resulting from fusion proteins bound to the cells was detected by

a fluorescent assay in 96-well FluoroNunc microplates. Cell extracts (25 µl) were mixed with 25 µl of 2× assay buffer containing 0.2 mM 4-methylumbelliferyl-phosphate (Sigma) in 0.1 M diethanolamine–HCl, pH 9.8, 1 mM MgCl$_2$. After 1 h of incubation at 37 °C, the reaction was stopped with 200 µl of 0.5 M glycine–NaOH. Fluorescence was read at 465 nm (emission) on an HTS 7000 Plus Bio Assay plate-reader (Perkin Elmer). To equalize loading, protein concentration was measured using a Lowry-modified colorimetric assay compatible with detergents (DC Protein Assay; BioRad).

Cell staining and immunohistochemistry. For histochemical staining, cells were grown to 80% confluence in six-well plates and incubated with the AP fusion proteins for 2 h at 25 °C. Cells were washed for 5 min on ice, four times with 2 ml of ice-cold HBSS and once with 2 ml of AP buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl). To inactivate endogenous AP, 1 mM levamisole (Sigma) was included in the last two washes. To develop AP activity, cells were incubated at 25 °C with 2 ml of a NBT (nitroblue tetrazolium chloride)/BCIP (5-bromo-4-chloro-3-indolyl-phosphate) solution filtered using a 0.2-µm filter (Roche Biochemicals) until positive purple cells were seen (Oelgeschläger *et al.*, 2000; Flanagan & Cheng, 2000).

For immunohistochemistry, COS-7 or 10T1/2 fibroblast cells were grown on coverslips coated with poly-L-lysine until 80% confluency was reached, incubated with Chd–Fc for 2 h on ice, and washed three times with ice-cold PBS. Some samples were incubated on ice and others at 37 °C for 30 min in PBS containing 0.5 mM Texas-Red-dextran (lysine fixable; molecular weight 3,000 Da; Molecular Probes) to mark endocytic vesicles (Entchev *et al.*, 2000). Cells were fixed with 4% paraformaldehyde in PBS for 20 min and permeabilized with 0.1% Triton X-100 for 10 min. Fluorescence was visualized after incubation with an anti-human IgG antibody conjugated to Alexa Fluor® 488 (1:200 dilution; Molecular Probes). For actin staining, Alexa Fluor® 568 phalloidin (1:200 dilution; Molecular Probes) was used after cell permeabilization.

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