lo^{Mut}, was assembled from two PCR fragments in which internal oligonucleotide primers were designed to introduce amino acid substitutions in the resultant zinc-binding domain. Subsequently, a PCR fragment encoding the COOH-terminal portion of zebrafish Tolloid was subcloned into pCS2:Metallo or pCS2:Metallo^{Mut}. The two resultant full-length constructs, pCS2:Tolloid and pCS2:Tolloid^{Mut}, were linearized with Not I and transcribed with the Message Machine Kit (Ambion). For transfection into COS cells, constructs were tagged at the COOH-terminus with the Myc epitope. The integrity of synthetic RNAs was assessed on agarose gels, and the RNAs were diluted in water to their respective final injection concentrations immediately before injection. Synthetic mRNAs were injected into the yolk of one- to two-cell-stage embryos with a gas-driven microinjector (Eppendorf).

24. J. S. Joly, C. Joly, S. Schultemerker, H. Boulekbache, H. Condamine, *Development* **119**, 1261 (1993).
25. H. W. Detrich *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10713 (1995).
26. S. Krauss, T. Johansen, V. Korzh, A. Fjose, *Development* **113**, 1193 (1991); A. W. Püschel, M. Westerfield, G. R. Dressler, *Mech. Dev.* **38**, 197 (1992).
27. A. L. Finelli, C. A. Bossie, T. Xie, R. W. Padgett, *Development* **120**, 861 (1994).
28. M. C. Mullins *et al.*, *ibid.* **123**, 81 (1996).
29. Epitope-tagged Chordin (Chd-MYC) was produced from a baculovirus construct with the use of High-Five cells (Invitrogen) (*9*), and Myc immunoreactivity was subsequently monitored by protein immunoblotting with monoclonal antibody to Myc (9E10) [G. L. Evans, G. K. Lewis, G. Ramsey, J. M. Bishop, *Mol. Cell. Biol.* **5**, 3610 (1985)]. Purified retinoid X receptor α–ligand binding domain (RXRα-LBD) was used as a control for protease specificity [W. Bourguet *et al.*, *Protein Express. Purif.* **6**, 604 (1995)]. COS cells were transfected with various DNA constructs with DEAE dextran as described [B. R. Cullen, *Methods Enzymol.* **152**, 684 (1987)]. Expression of the transfected constructs was monitored in parallel by immunohistocytochemistry. The proteolytic potential of the transfected cells was assayed after 48 hours in culture by removing half of the growth media from the COS cells and replacing it with an equal volume of prewarmed baculovirus extract supplemented with RXRα-LBD. Samples of the resulting media were removed at various times after addition of the substrates and were assayed for immunoreactivity by protein immunoblotting. Proteins from the transfected constructs were expressed at a level insufficient for detection by protein immunoblotting of the COS supernatants.

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Role of Inositol 1,4,5-Trisphosphate Receptor in Ventral Signaling in *Xenopus* Embryos

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The inositol 1,4,5-trisphosphate (IP_3) receptor is a calcium ion channel involved in the release of free Ca^{2+} from intracellular stores. For analysis of the role of IP_3-induced Ca^{2+} release (IICR) on patterning of the embryonic body, monoclonal antibodies that inhibit IICR were produced. Injection of these blocking antibodies into the ventral part of early *Xenopus* embryos induced modest dorsal differentiation. A close correlation between IICR blocking potencies and ectopic dorsal axis induction frequency suggests that an active IP_3-Ca^{2+} signal may participate in the modulation of ventral differentiation.

In development, the formation of the body plan involves receptor-mediated signal transduction in processes such as mesoderm and neural induction. Activation of the polyphosphoinositide (PI) cycle results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate, producing IP_3 and diacylglycerol (*1*). IP_3 triggers the release of Ca^{2+} from the endoplasmic reticulum into the cytosol through the IP_3 receptor (IP3R) (*2*). The PI cycle has been postulated to function in dorso-ventral (D-V) axis formation in many species, as indicated by the action of lithium (*3*, *4*). Lithium is assumed to block the recycling of IP_3 into inositol by inhibiting the hydrolysis of intermediate inositol phosphates (*3*). Application of lithium to cleavage-stage embryos of *Xenopus laevis* induces dorsalization by conversion of ventral mesoderm to dorsal mesoderm, with a concomitant reduction in posterior structures (*4*). Although some studies suggest that IP_3 functions in transducing ventral signals during mesoderm induction (*5*), other work does not support the inositol depletion hypothesis for the action of lithium (*6*, *7*).

To determine the role of the PI cycle in patterning the body plan, we isolated monoclonal antibodies (mAbs) to the *Xenopus* IP_3 receptor (XIP3R) (*8*, *9*) and analyzed their effects on D-V specification. Protein immunoblot analysis revealed that mAbs 1G9, 11B12, and 7F11 recognized a single band of the same molecular size as XIP3R (*10*) (Fig. 1A). Antibodies 1G9 and 11B12 inhibited IP_3-induced Ca^{2+} release (IICR) in an in vitro assay (*11*) (Fig. 1B). To confirm the inhibitory activity of the mAbs in vivo, we assessed the effects of the mAbs on early *Xenopus* embryos that overexpressed an exogenous PI-coupled receptor, the type I muscarinic acetylcholine receptor (m1AChR) (*12*, *13*) (Fig. 1C). Although 7F11 did not inhibit IICR in the in vitro Ca^{2+} release assay, all three mAbs had inhibitory activity in vivo. In the presence of mAbs 1G9, 11B12, or 7F11, Ca^{2+} release upon ligand application was 26.9, 10.8, or 58.6%, respectively, of that observed in control embryos.

To examine the role of IICR on axis formation, we injected the blocking antibodies into either ventral or dorsal blastomeres. Ventral injection of 1G9, 11B12, or 7F11 at the four-cell stage (*14*) induced the formation of a secondary dorsal axis, whereas dorsal injection (*15*) of the mAbs or normal mouse immunoglobulin G (IgG) (Fig. 2, A and B) showed no obvious effects. When a low dose (20 ng per embryo) of 1G9 or 11B12, or a higher dose of 7F11 (40 ng per embryo), was applied, the induced axes consisted of ectopic muscle and neural structures, but usually lacked notochords (Fig. 2C). At a higher dose of 1G9 (80 ng per embryo), hyperdorsoanteriorization occurred at the expense of dorsoposterior development (*15*). Dose responses of the mAbs are presented in Fig. 2D. The relative potency of the ectopic axis–inducing activity of the mAbs corresponded to their abil-

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ity to block IICR: 11B12 ≈ 1G9 > 7F11. A dose of 7F11 (40 ng per embryo), which effectively induced ectopic axis (Fig. 2D), reduced IICR to about 58.6% of the control (Fig. 1D). Thus, a partial reduction in IICR elicits a respecification of ventral mesoderm. Co-injection of 1G9 with a 12-residue-long peptide of the sequence GQPAHLNINPQQ (*16*) resulted in a complete rescue of the phenotype (Fig. 2, E and F), further confirming the specificity of 1G9. However, we have been unable to show that the restoration of Ca^{2+} influx can rescue the effect of the mAbs because of technical reasons (*15*). Therefore, we cannot completely exclude the possibility that the effect of the antibodies is independent of IICR.

Because injection of the blocking mAbs of the IICR seemed to modify the response of mesoderm to mesodermal inducers, we examined the effect of the mAbs on marginal zone explants (*17*). Upon injection of 1G9, the ventral marginal zone (VMZ) explants became morphologically indistinguishable from the dorsal marginal zone (DMZ) explants, which elongated in culture (*15*). Reverse transcriptase–polymerase chain reaction (RT-PCR) studies revealed that in VMZ explants, 1G9 injection induced the expression of both early and late dorsal markers (Fig. 3) (*18*). In early gastrula stages, expression of the secreted organizer proteins encoded by the genes *chordin* (*19*), *follistatin* (*20*), and *noggin* (*21*) as well as expression of α-*actin* (*22*) was induced in the 1G9-injected VMZs. A marker for dorsal mesoderm, *goosecoid* (*23*), was slightly induced, whereas *Xnot1* (*24*), a marker for notochord, was not induced in the VMZs (*15*). The expression of a ventral-specific homeobox gene *Xvent-1* (*25*) was inhibited in the VMZs that were injected with 1G9 (Fig. 3B). In the later stages (neurula stage 20), a similar conversion of VMZs to DMZs was also evident. Expression of the neural cell adhesion molecule (*NCAM*) (*26*) and the dorsal mesoderm marker α-*actin* were turned on in VMZs injected with 1G9. Thus, the IP_3-Ca^{2+} signaling system may transduce ventral signals in patterning early embryos, and inhibition of this ventral signal leads to dorsal differentiation.

We then examined whether injection of antibody to XIP3R would restore the dorsal axis in embryos that were ventralized by ultraviolet (UV) irradiation (*4*). A partial rescue of dorsal structures was observed in embryos injected with 1G9; although the most anterior head structures were missing, a substantial rescue of trunk and tail structures had occurred (Fig. 4A). Blockage of the IP_3-Ca^{2+} signaling system revealed an underlying capacity of the embryo to form dorsal structures. We next examined how

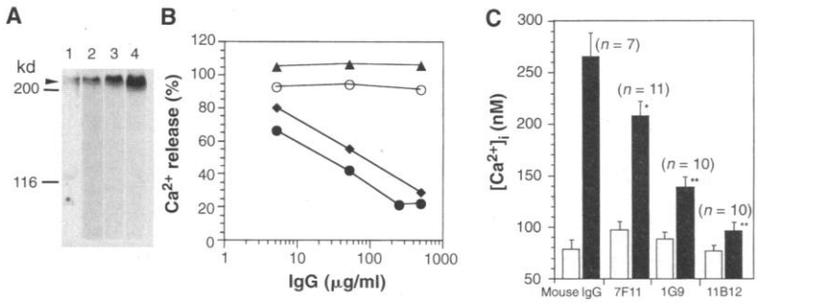

Fig. 1. Isolation of XIP3R-specific mAbs with IICR blocking activity. (**A**) The mAbs 1G9 (lane 2), 11B12 (lane 3), and 7F11 (lane 4) at a final concentration of 40 μg/ml specifically recognize XIP3R as determined by protein immunoblot analysis [done as described in (*10*)]. An XIP3R-specific polyclonal antibody was used as a positive control (lane 1). Whole-cell clear lysates (*8*) equivalent to one unfertilized *Xenopus* egg were loaded into each lane of a 5% SDS-polyacrylamide gel. (**B**) The mAbs 1G9 and 11B12 effectively inhibited IICR in an in vitro IICR assay. Symbols: 1G9 (●); 11B12 (◆); 7F11 (▲); and control mouse IgG (○). (**C**) XIP3R mAbs inhibited IICR in vivo. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in animal pole blastomeres before (white bars) and after (black bars) carbachol applications are shown as means ± SEM. The differences were significant for animal blastomeres injected with XIP3R mAbs compared with the control mouse IgG–injected animal blastomeres (*P < 0.05 for 7F11 and **P < 0.005 for 1G9- and 11B12-injected animal blastomeres, analyzed by two-tailed, paired Student's t test).

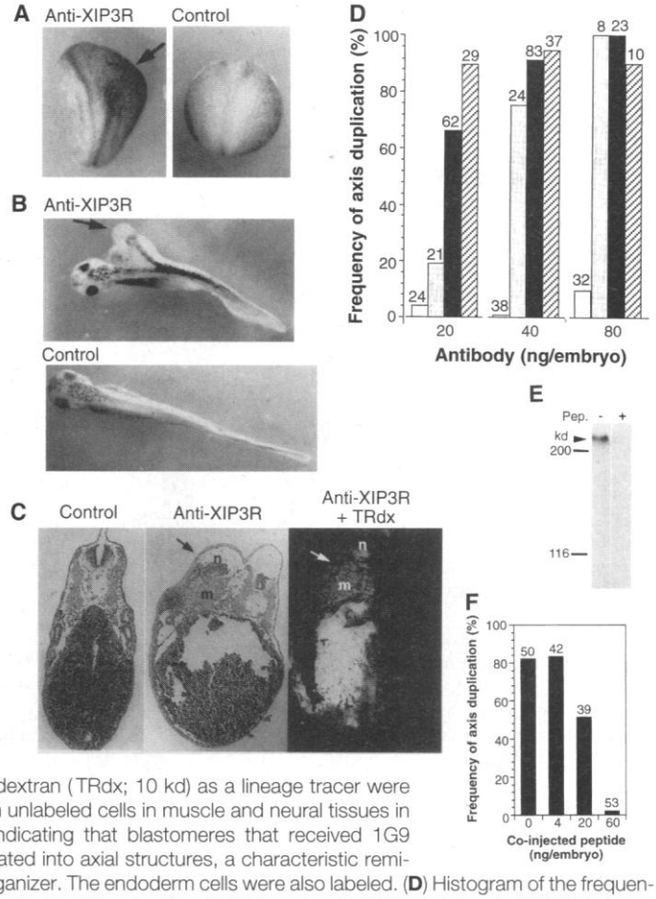

Fig. 2. Dorsal duplication induced by ventral injection of mAb 1G9. (**A**) Embryos (dorsal view) at stage 20 that were injected ventrally at the four-cell stage with 40 ng per embryo of control mouse IgG or 1G9 (anti-XIP3R). The induced ectopic dorsal axis is indicated by an arrow. (**B**) Embryos injected ventrally at the four-cell stage with 40 ng per embryo of control mouse IgG or 1G9. Dorsal views of embryos at stage 37/38 are shown. A partial duplication of the dorsal axis (arrow) can be observed in 1G9-injected embryos. (**C**) Transverse sections of a control embryo injected with mouse IgG or 1G9. Note the presence of ectopic neural (n) and muscle tissues (m) in embryos injected with 1G9. Arrows indicate the ectopic dorsal axis. Cells labeled with dextran Texas Red lysine dextran (TRdx; 10 kd) as a lineage tracer were observed intermingled with unlabeled cells in muscle and neural tissues in the ectopic dorsal axis, indicating that blastomeres that received 1G9 injections directly differentiated into axial structures, a characteristic reminiscent of the Spemann organizer. The endoderm cells were also labeled. (**D**) Histogram of the frequency of axis duplication induced with different mAbs: 7F11 (gray bars), 1G9 (black bars), 11B12 (hatched bars), or control mouse IgG (white bars). The numbers of embryos examined are shown above the bars. (**E**) Protein immunoblot analysis with 1G9 (40 μg/ml) shows that the band corresponding to XIP3R (arrowhead) was completely competed out by the 12-residue-long peptide (GQPAHLNINPQQ) (*15*) at 40 μg/ml. Each lane contains one unfertilized-egg-equivalent of whole-cell clear lysate. (−), 1G9 without the peptide (Pep.); (+), 1G9 with the peptide. (**F**) The frequency of axis duplication induced by 1G9 (20 ng per embryo) decreased when 1G9 was co-injected with increasing amounts of the competing peptide. Numbers of embryos examined are above the bars.

the IP_3 signaling system would act with other ventralizing signals. When overexpressed in the early embryo, bone morphogenetic protein 4 (BMP4) causes ventralization of embryos (*27*). This ventralization was suppressed when 1G9 was coinjected with BMP4 RNA (*28*) into dorsal blastomeres of four-cell stage embryos (Fig. 4B) (*29*). This finding further confirmed that IP_3-Ca^{2+} signaling is a component of the ventralizing signal.

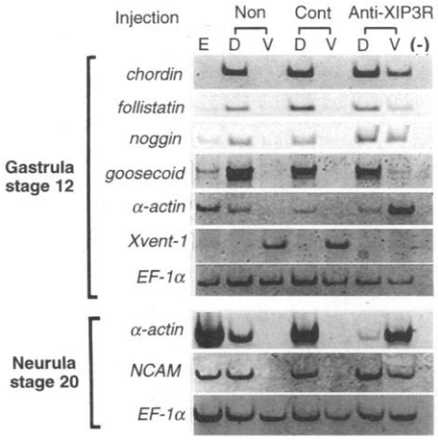

Fig. 3. Respecification of VMZs to DMZs by 1G9. Injection of 1G9 (anti-XIP3R) changes the molecular characteristics of VMZs to those of DMZs. RNA from the explants was analyzed by RT-PCR for the presence of *goosecoid*, *α-actin*, *Xvent-1*, *NCAM*, and *EF-1α* (*33*). *EF-1α* is a loading control. Non, uninjected; Cont, mouse IgG injected. Lane E, whole embryos; lane D, DMZs; lane V, VMZs; (−), a negative control that contains no reverse transcriptase.

To determine if injection of antibody to XIP3R would induce mesodermal differentiation and whether antibody injection would interfere with activin- or basic fibroblast growth factor (bFGF)–mediated mesoderm induction (*30*), we investigated its effects on animal cap explants. Animal caps injected with 1G9 alone did not express dorsal mesodermal markers such as *Xbra* (*31*) or *α-actin* or the neural marker *NCAM* (Fig. 4C). Animal caps injected with 1G9 responded normally to activin A or bFGF, as indicated by expression of *Xbra*, *α-actin*, and *NCAM*. Thus, inhibition of the IP_3-Ca^{2+} signaling system alone is insufficient to induce mesodermal tissues, and inhibition of the IP_3-Ca^{2+} signaling does not block activin or bFGF signaling in animal caps.

Our results that injection of antibody to XIP3R cannot induce head mesoderm resembles observations made in grafting experiments of the gastrula-stage organizer (*32*). Injection of the antibody might mimic the late activity of the organizer that can rescue neural tissue and somites but not notochord or head structures. In fact, expression of the secreted organizer proteins encoded by *chordin*, *noggin*, and *follistatin* (Fig. 3B), and a slight expression of the organizer-specific homeobox gene *goosecoid* but not *Xnot1*, is induced in anti-XIP3R–injected VMZs. Therefore, blockage of the IP_3-Ca^{2+} signaling system is sufficient for dorsal respecification to take place, but not for the induction of a full spectrum of the organizer-specific genes. Additional factors are required for induction of the early organizer.

Inhibition of IICR in *Xenopus* embryos partially mimicked the effect of lithium. However, the extent of dorsalization differs between the effect of lithium and that of anti-XIP3R. Lithium treatment causes a duplication of a complete secondary axis, whereas anti-XIP3R injection induced only a posterior axis (Fig. 2). Lithium can completely rescue dorsal structures in UV-irradiated ventralized embryos, whereas only a partial rescue occurs with 1G9 (Fig. 4A). Lithium but not anti-XIP3R can sensitize the response of animal cap cells to bFGF (Fig. 4C). Thus, the effect of lithium may result from the combined inhibition of both the IP_3-Ca^{2+} signaling system and other targets such as glycogen synthase kinase-3 (*7*).

Blockage of IICR in the ventral part of *Xenopus* embryos converted ventral mesoderm to dorsal mesoderm, thereby generating an ectopic dorsal axis. The correlation between IICR blocking potencies of mAbs and ectopic dorsal axis induction suggests that the activity of the IP_3-Ca^{2+} signaling system regulates D-V patterning.

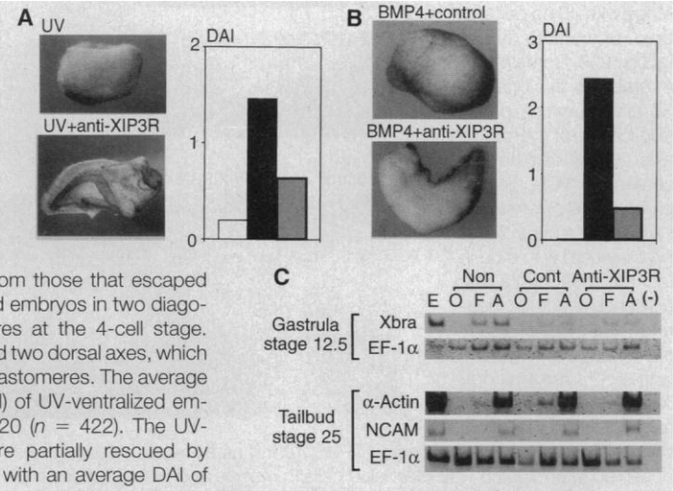

Fig. 4. (**A**) The mAb 1G9 can rescue the dorsal axis in UV-irradiated ventralized embryos. A typical UV-ventralized embryo (UV) and a UV-treated embryo rescued by 1G9 injection (UV + anti-XIP3R) are shown at an equivalent stage of 33/34. To distinguish 1G9-rescued embryos from those that escaped UV treatment, we injected embryos in two diagonally opposed blastomeres at the 4-cell stage. These embryos developed two dorsal axes, which arose from the injected blastomeres. The average dorsal anterior index (DAI) of UV-ventralized embryos (white bar) was 0.20 (n = 422). The UV-ventralized embryos were partially rescued by 1G9 injection (black bar) with an average DAI of 1.46 (n = 79), whereas control UV-ventralized embryos injected with mouse IgG (gray bar) had an average DAI of 0.64 (n = 11). (**B**) Injection of 1G9 reversed the ventralizing activity of BMP4 RNA. We co-injected 40 ng per embryo of 1G9 (BMP + anti-XIP3R) or control mouse IgG (BMP + control) with 1 ng of BMP4 RNA (per embryo) into two dorsal blastomeres of 4-cell stage embryos. Co-injection of 1G9 suppressed the ventralizing effect of BMP4. BMP4 RNA injection (white bar) gave an average DAI of 0 (n = 23), co-injection of 1G9 with BMP4 RNA (black bar) gave an average DAI of 2.43 (n = 22), and co-injection of control mouse IgG with BMP4 RNA (gray bar) gave an average DAI of 0.5 (n = 18). (**C**) Injection of 1G9 alone is not sufficient for mesoderm induction. Embryos were injected with 80 ng per embryo of 1G9 or control mouse IgG (cont) into the animal poles of both blastomeres at the 2-cell stage. Lane E, whole embryos; lanes 0, no growth factors; lanes F, bFGF (100 ng/ml) (BRL); lanes A, activin A (50 ng/ml); (−), a negative control that contains no reverse transcriptase; Non, uninjected.

REFERENCES AND NOTES

1. M. J. Berridge, *Biochem. J.* **220**, 345 (1984).
2. C. D. Ferris, R. L. Huganir, S. Supattapone, S. H. Snyder, *Nature* **342**, 87 (1989); N. Maeda, M. Niinobe, K. Mikoshiba, *EMBO J.* **9**, 61 (1990).
3. M. J. Berridge, C. P. Downes, M. R. Hanley, *Cell* **59**, 411 (1989).
4. K. R. Kao, Y. Masui, R. P. Elinson, *Nature* **322**, 371 (1986); J. Cooke and E. J. Smith, *Development* **102**, 85 (1988); K. R. Kao and R. P. Elinson, *Dev. Biol.* **132**, 81 (1989); S. E. Stachel, D. J. Grunwald, P. Z. Myers, *Development* **117**, 1261 (1993).
5. Inositol rescues *Xenopus* embryos from the teratogenic effects of lithium [W. B. Busa and R. L. Gimlich, *Dev. Biol.* **132**, 315 (1989)]. There is an increase in the total amount of embryonic IP_3 during the early blastula stage of *Xenopus* embryos and a decrease of IP_3 after lithium treatment [J. A. Maslanski, L. Leshko, W. B. Busa, *Science* **256**, 243 (1992)], although the increase in IP_3 that we detected first occurred at early gastrula stage [S. Kume, A. Muto, H. Okano, K. Mikoshiba, *Mech. Dev.* **66**, 157 (1997)]. Dorsal activation of the PI cycle–stimulating receptor, 5-hydroxytryptamine (HT_{1C}) receptor, blocked dorsal development [K. T. Ault, G. Durmowicz, A. Galione, P. L. Harger, W. B. Busa, *Development* **122**, 2033 (1996)].
6. Ventral injection of an antagonist of inositol monophosphatase, bisphosphonate L-690,330, which is more potent than lithium, failed to induce an ectopic dorsal axis in *Xenopus* embryos [J. R. Atack, S. M. Cook, A. P. Watt, S. R. Fletcher, C. I. Ragan, *J. Neurochem.* **60**, 652 (1993)].
7. P. S. Klein, D. A. Melton, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8455 (1996).
8. S. Kume *et al.*, *Cell* **73**, 555 (1993).
9. To prepare an antibody that could block IICR specifically, we screened mAbs to the 155 amino acids at

the COOH-terminus of the XIP3R protein (*8*). The mAbs to XIP3R were isolated by fusion of the P3U1 myeloma cell line P3x63AgU1 with spleen cells derived from BALB/c mice immunized with glutathione-S-transferase–XIP3R fusion protein. Initial screenings were done by enzyme-linked immunosorbent assays (ELISA) with TrpE-XIP3R fusion proteins. The serum-free culture supernatant of hybridoma cell lines and commercial mouse IgG used as controls were affinity purified with Ampure IgG purification kits (Amersham), followed by dialysis against injection buffer (88 mM NaCl, 1 mM KCl, 15 mM tris-HCl, pH 7.5). With the assumption that the volume of the 2-cell stage embryo is 1 μl [Y. Chen, L. Huang, M. Solursh, *Dev. Biol.* **161**, 70 (1994)], injection of antibody at 20 ng per embryo into the VMZs of 4-cell stage embryos resulted in the estimated final concentration of mAb of ~40 μg/ml.

10. S. Kume *et al.*, *Dev. Biol.* **182**, 228 (1997).
11. The release of Ca^{2+} from microsomes of *Xenopus* oocytes by the addition of 100 nM IP_3 was measured. The amount of IICR was normalized to the amplitude of the Ca^{2+} release in the presence of control mouse IgG [A. Muto, S. Kume, T. Inoue, H. Okano, K. Mikoshiba, *J. Cell Biol.* **135**, 181 (1996)].
12. K. Fukuda *et al.*, *Nature* **327**, 623 (1987).
13. The Ca^{2+} indicator dye Fura 2 (Molecular Probe) (20 μM) and m1AChR mRNA (13 ng per embryo) were micro-injected at the 2-cell stage, and then mAb (40 ng per embryo) was injected at the animal poles. Animal pole blastomeres were cut off between the 32- to 128-cell stage. Changes in the fluorescence intensities excited at 340 and 380 nm upon carbachol addition (100 μM) were recorded and analyzed.
14. P. D. Nieuwkoop and J. Faber, *The Normal Development Table of* Xenopus laevis (North-Holland, Amsterdam, 1967).
15. S. Kume, unpublished observation.
16. To obtain more precise information about the sequence that the inhibitory mAbs recognize, we synthesized a candidate epitope of a 12-residue-long peptide with the sequence GQPAHLNINPQQ (corresponding to residues 2680 to 2691, at the COOH terminal of XIP3R) (*8*) (Peptide Institute). Both ELISA and protein immunoblot analysis revealed that binding of the mAbs 11B12 and 1G9, but not 7F11, to XIP3R was inhibited in the presence of this peptide (*15*). Abbreviations for the amino acid residues are as follows: A, Ala; G, Gly; H, His; I, Ile; L, Leu; N, Asn; P, Pro; and Q, Gln.
17. Embryos were injected with 80 ng per embryo of 1G9 or control mouse IgG into the marginal zones of each blastomere at the 2-cell stage. DMZs or VMZs were explanted from stage 10 to 10.5 early gastrula embryos and cultured in 1× Steinberg's solution.
18. Total RNA was isolated from 10 explants. Reverse transcription was carried out with oligo(dT) primers. The primer pairs we used were described previously (*19–23*, *25*, *26*). RT-PCR products were stained with DNA-sensitive dye, SYBR Green I, and analyzed by FluorolmagerTM (Molecular Dynamics).
19. Y. Sasai, *et al.*, *Cell* **79**, 779 (1994); Y. Sasai, B. Lu, H. Steinbeisser, E. M. De Robertis, *Nature* **376**, 333 (1995).
20. A. Hemmati-Brivanlou, O. G. Kelly, D. A. Melton, *Cell* **77**, 283 (1994).
21. W. C. Smith and R. M. Harland, *ibid.* **70**, 829 (1992); W. C. Smith, A. K. Knecht, M. Wu, R. M. Harland, *Nature* **361**, 547 (1993).
22. F. Stutz and G. Spohr, *J. Mol. Biol.* **187**, 349 (1986).
23. K. W. Cho, B. Blumberg, H. Steinbeisser, E. M. De Robertis, *Cell* **67**, 1111 (1991).
24. G. Von Dassow, J. E. Schmidt, D. Kimelman, *Genes Dev.* **7**, 355 (1993).
25. V. Gawantka, H. Delius, K. Hirschfeld, C. Blumenstock, C. Niehrs, *EMBO J.* **14**, 6268 (1995).
26. C. R. Kintner and D. A. Melton, *Development* **99**, 311 (1987).
27. C. M. Jones, K. M. Lyons, P. M. Lapan, C. V. E. Wright, B. J. M. Hogan, *ibid.* **115**, 639 (1992); L. Dale, G. Howes, B. M. J. Price, J. C. Smith, *ibid.*, p. 573.
28. S. Nishimatsu, A. Suzuki, A. Shoda, K. Murakami, N. Ueno, *Biochem. Biophys. Res. Commun.* **186**, 1487 (1992).
29. Our result that the expression of the ventral homeobox gene *Xvent-1*, a downstream component of BMP4 (*24*), was eliminated by 1G9 injection suggests that the expression of *Xvent-1* may be positively regulated by the IP_3-Ca^{2+} signaling system. Our present results show that the inhibition of the IP_3-Ca^{2+} signaling pathway induced the expression of *noggin* and *chordin* genes (Fig. 3). The up-regulation of these BMP4 antagonists by 1G9 injection suggests a negative regulation of these genes by the IP_3-Ca^{2+} signaling system, either directly or through negative cross-regulatory loops, as described between *Xvent-2* and *goosecoid* [D. Onichtchouk *et al.*, *Development* **122**, 3045 (1996)]. As for the upstream factors that may activate the IP_3-Ca^{2+} signaling system, Xwnt-5A is a candidate [D. C. Slusarski, J. Yang-Snyder, W. B. Busa, R. T. Moon, *Dev. Biol.* **182**, 114 (1997)].
30. G. Thomsen *et al.*, *Cell* **63**, 485 (1990); M. Asashima *et al.*, *Roux's Arch. Dev. Biol.* **198**, 330 (1990); J. M. W. Slack, B. G. Darlington, J. K. Heath, S. F. Godsave, *Nature* **326**, 197 (1987); Y. Eto *et al.*, *Biochem. Biophys. Res. Commun.* **142**, 1095 (1987).
31. J. C. Smith, B. M. Price, J. B. Green, D. Weigel, B. G. Herrmann, *Cell* **67**, 79 (1991).
32. J. C. Smith and J. M. Slack, *J. Embryol. Exp. Morphol.* **78**, 299 (1983); L. A. Lettice, J. M. W. Slack, *Development* **117**, 263 (1993).
33. P. Krieg, S. Varnum, M. Woormington, D. A. Melton, *Dev. Biol.* **133**, 93 (1989).
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Crystal Structure of the Adenylyl Cyclase Activator $G_{s\alpha}$

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The crystal structure of $G_{s\alpha}$, the heterotrimeric G protein α subunit that stimulates adenylyl cyclase, was determined at 2.5 Å in a complex with guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS). $G_{s\alpha}$ is the prototypic member of a family of GTP-binding proteins that regulate the activities of effectors in a hormone-dependent manner. Comparison of the structure of $G_{s\alpha}$·GTPγS with that of $G_{i\alpha}$·GTPγS suggests that their effector specificity is primarily dictated by the shape of the binding surface formed by the switch II helix and the α3-β5 loop, despite the high sequence homology of these elements. In contrast, sequence divergence explains the inability of regulators of G protein signaling to stimulate the GTPase activity of $G_{s\alpha}$. The βγ binding surface of $G_{s\alpha}$ is largely conserved in sequence and structure to that of $G_{i\alpha}$, whereas differences in the surface formed by the carboxyl-terminal helix and the α4-β6 loop may mediate receptor specificity.

The G_s and G_i subfamilies of heterotrimeric G protein α subunits, although highly homologous, differ profoundly with respect to effector, regulator, and receptor specificity (*1*, *2*). For example, $G_{s\alpha}$ binds to and activates all isoforms of adenylyl cyclase (*3*), whereas $G_{i\alpha 1}$ and its close paralogs inhibit only certain isoforms of the effector. The GTPase activities of G_i subfamily members are stimulated by members of the RGS (regulators of G protein signaling) protein family; the GTPase activity of $G_{s\alpha}$ is not affected by any known RGS protein (*4*). Distinct subfamilies of G protein–coupled receptors activate either G_s or G_i. To better understand the origins of these functional differences, we have determined the three-dimensional structure of GTPγS-activated $G_{s\alpha}$ alone and in complex with its effector, adenylyl cyclase (*5*). Comparison of the structure of $G_{s\alpha}$ with those of previously determined G_i subfamily members (*6*, *7*) offers substantial insight into the molecular basis of specificity in heterotrimeric G proteins.

Deficiencies in $G_{s\alpha}$ function have serious biological repercussions. Adenosine diphosphate (ADP) ribosylation of the active site residue Arg^{201} by cholera toxin (*8*, *9*) leads to irreversible inhibition of the GTPase activity of $G_{s\alpha}$. The resulting constitutive activation of adenylyl cyclase in gastrointestinal epithelium is responsible for the diarrhea and dehydration that are the hallmarks of cholera. Similarly, mutation of Arg^{201} or the catalytic residue Gln^{227} contributes to the growth of tumors of the pituitary and thyroid glands and causes the McCune-Albright syndrome (*10*, *11*). Heterozygous deficiency of $G_{s\alpha}$ is the basis for pseudohypoparathyroidism (type IA) (*11–13*).

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