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18. Mouse L cells (Cc2) expressing bovine CI-MPR were grown to confluency on 15-cm tissue culture dishes in α-MEM (Mediatech) and 10% fetal bovine serum. The cells were washed twice with phosphate-buffered saline, scraped into assay buffer containing 0.2% Triton X-100, and sonicated. After centrifugation at 15,000*g*, the supernatant was used immediately in binding assays (*17*). GST-GGA2 fusion constructs were made by polymerase chain reaction (PCR) with GST-GGA2(1-613)pGEX4T (*6*) as template DNA and ligation into the vector pGEX6P (Amersham Pharmacia). Fusion proteins were expressed and purified as described (*17*).
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20. The construction of the deletion mutants (Dd4, Ee5, Ff3, and 344A) and the AC mutants and their effect on sorting are described elsewhere (*2*, *3*, *5*).
21. GST fusion protein constructs encoding the wild-type bovine 163–amino acid cytoplasmic tail and the deletion or point mutants were made by PCR as described (*17*). HA-GGA2(1–613)pRK5 was transfected into COS cells (*6*), and the cell lysate was prepared as with mouse L cells (*18*). GST pull-down assays were performed with either bovine brain cytosol (AP-1) or the COS cell lysate (HA-GGA2).
22. Site-directed mutagenesis was carried out with primers incorporating the desired mutations with the QuickChange system (Stratagene). GST pull-down assays were performed with rat liver cytosol as the source of soluble clathrin. The TD.1 monoclonal antibody was a gift from F. Brodsky
23. We thank P. Lobel for providing many cell lines for this study; K. and R. Kornfeld, D. Tollefsen, D. Ory, and M. Drake for helpful comments; and J. Bonifacino for sharing unpublished data. This work was supported in part by NIH grant RO1 CA-08759 to S.K.

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G Protein Signaling from Activated Rat Frizzled-1 to the β-Catenin–Lef-Tcf Pathway

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The *frizzled* receptors, which mediate development and display seven hydrophobic, membrane-spanning segments, are cell membrane–localized. We constructed a chimeric receptor with the ligand-binding and transmembrane segments from the β_2-adrenergic receptor (β_2AR) and the cytoplasmic domains from rat Frizzled-1 (Rfz1). Stimulation of mouse F9 clones expressing the chimera (β_2AR-Rfz1) with the β-adrenergic agonist isoproterenol stimulated stabilization of β-catenin, activation of a β-catenin–sensitive promoter, and formation of primitive endoderm. The response was blocked by inactivation of pertussis toxin–sensitive, heterotrimeric guanine nucleotide–binding proteins (G proteins) and by depletion of Gαq and Gαo. Thus, G proteins are elements of Wnt/Frizzled-1 signaling to the β-catenin–lymphoid-enhancer factor (LEF)-T cell factor (Tcf) pathway.

Wnts constitute a family of vertebrate genes encoding ligands essential to signaling in early development, signaling that includes control of cell proliferation, cell fate, and embryonic patterning (*1*). These secreted glycoproteins act via members of the *frizzled* gene family (*2–4*). Signaling downstream of some Frizzled homologs in response to Wnt-1 or Wnt-8 leads to activation of the phosphoprotein Dishevelled (Dsh/Dvl), which then represses the function of glycogen synthase kinase-3β (GSK-3) activity (*5*, *6*). In the absence of Wnt, GSK-3 phosphorylates β-catenin, reducing its stability and abundance. Wnt signaling represses GSK-3 activity, thereby increasing the stability and intracellular accumulation of β-catenin, which then accumulates in the nucleus where it binds to members of the Lef-Tcf classes of architectural high-mobility group box transcription factors to activate genes involved in early development. Analysis of this pathway has been hindered by the lack of easy experimental methods for tightly controlling the receptor activation state or inhibition of the receptor.

To allow the rapid activation and inhibition of Frizzled coupled to the β-catenin pathway, we created a chimeric receptor consisting of the extracellular and transmembrane segments of the hamster β_2AR and the cytoplasmic domains of the Rfz1 (Fig. 1A). The sequence of the Rfz1 cytoplasmic domains diverge from those of the β_2AR (*7*, *8*). This chimeric receptor has the potential to be activated by soluble drugs of well-known pharmacology. Mouse F9 teratocarcinoma cells were stably transfected with an expression vector harboring the β_2AR-Rfz1 chimera (*9*). Clones expressing mRNA encoding the Rfz-1 chimeric receptor in large amounts were identified by reverse transcription–polymerase chain reaction (RT-PCR) and propagated (Fig. 1B). Expression of the chimeric receptor was quantified readily using labeled iodocyanopindolol (ICYP), a high-affinity β-adrenergic antagonist ligand that binds specifically to the transmembrane domain of the β_2AR. ICYP-binding studies of Chinese hamster ovary (CHO) clones (which do not express endogenous β_2AR) stably transfected with pβ_2AR-Rfz1 vector demonstrate a K_d of ~80 pM and a maximal binding capacity (B_{max}) of 2 to 4 pmol ICYP binding per milligram of protein (*10*, *11*). Immunoblots of cell membranes from F9 clones expressing the β_2AR-Rfz1 chimera stained with antibodies to an extracellular epitope of the β_2AR identified the endogenous 65-kD β_2AR and a 55-kD molecular species with the predicted size of the chimera (Fig. 1C). ICYP binding to the β_2AR-Rfz1 chimera displays a rightward shift of the affinity of the chimera for the β-adrenergic agonist isoproterenol (ISO) in the presence of a GTP analog (GTP-γ-S, Fig. 1D). All heptihelical receptors known to operate via heterotrimeric G proteins display this characteristic GTP-dependent shift in agonist affinity (*12*).

Before testing the signaling activity of β_2AR-Rfz1, we needed an assay for signaling by Rfz1. F9 cells stably transfected to express wild-type Rfz1 form primitive endoderm (PE) when treated with conditioned medium containing Xwnt-8, as monitored by positive staining for the PE markers cytokeratin endo-A (i.e., antigen for the TROMA monoclonal antibody) and tissue plasminogen activator (*13*, *14*). Clones expressing the β_2AR-Rfz1 chimera were therefore treated with the β-adrenergic agonist ISO or the β-adrenergic antagonist propranolol, or both. Isoproterenol stimulated formation of PE in stably transfected F9 stem cells and propranolol blocked this response (Fig. 2A). Taken together these data demonstrate the ability of the Rfz1 chi-

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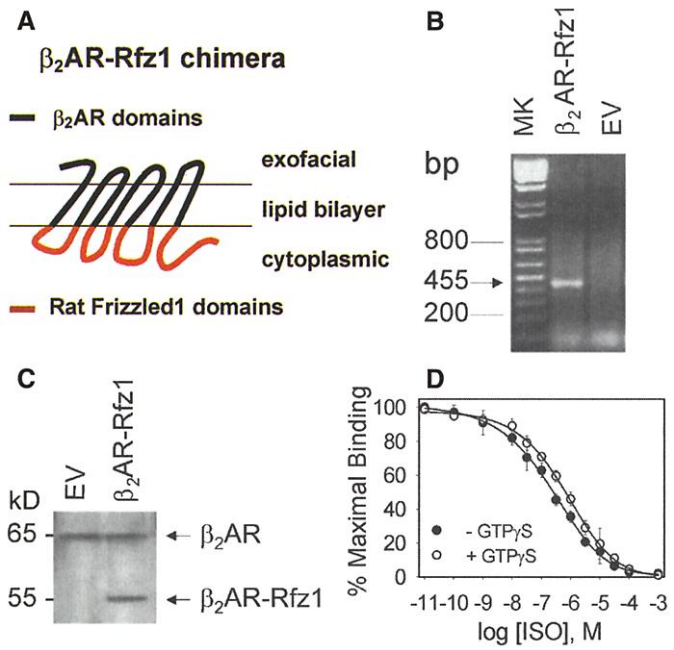

Fig. 1. Design and expression of a β_2AR-Rfz1 chimera in F9 stem cells. (**A**) Schematic of the chimera. (**B**), RT-PCR of β_2AR-Rfz1 mRNA expression in F9 clones. The RNA of F9 clones harboring either the empty expression vector (EV) or the vector expressing the β_2AR-Rfz1 chimera were reverse transcribed and amplified. Two primers, CCGGCCTTACCTCCT-TCTTGCCC and CCGT-TCTGCGACTTGAGCAC-CTCC, were employed in the PCR amplification. The molecular markers (Mk) indicate the relative size in base pairs (bp) of the amplified products. (**C**) Immunoblotting of immunoprecipitates of F9 clones stably transfected with either empty vector (EV) or vector expressing the β_2AR-Rfz1 chimera. Blots were stained with an antibody to an exofacial domain of the β_2AR, making visible both the native β_2AR of F9 cells (65 kD) as well as the β_2AR-Rfz1 chimeric receptor species (55 kD). (**D**) Dose-response curve for inhibition of ICYP binding by the β-adrenergic agonist isoproterenol, performed in the absence (−) and presence (+) of 10 μM GTPγS. The results are mean values ±SEM from six determinations.

mera to be stably expressed, to bind β-adrenergic ligands with normal pharmacological properties, to display a GTP-dependent agonist shift of affinity, and to signal to PE formation.

Because PE formation in culture measured above requires 3 to 4 days, we also tested whether activation of the wild-type Rfz1 in F9 cells would more rapidly induce the Lef- and Tcf-sensitive luciferase reporter of β-catenin signaling, pTOPFLASH (*15, 16*). Conditioned medium containing Xwnt-8, but not Xwnt-5a or empty vector EV, stimulated TOPFLASH expression (Fig. 2B). Wnt-5a signals via Rfz2 in a manner that is largely independent of β-catenin (*5*). Xwnt-5a stimulation of clones expressing rat Frizzled-2 (Rfz2) did not induce pTOPFLASH. We also measured the effect of ISO on the activation of the β-catenin–Lef-Tcf pathway in clones expressing the β_2AR-Rfz1 (Fig. 2C). Transcription of pTOPFLASH reporter was increased in response to ISO in clones expressing the chimera, but not clones harboring either the mutated pFOPFLASH, a control luciferase reporter vector lacking the Lef-Tcf–sensitive elements, or the empty vector. The β-adrenergic antagonist propranolol (PROP) did not activate the transcription

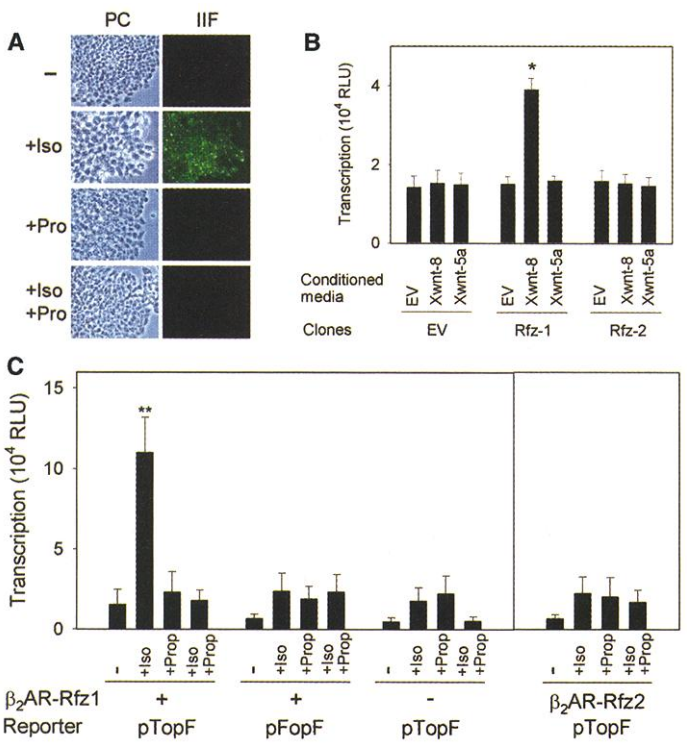

Fig. 2. ISO-stimulated formation of primitive endoderm and activation of the β-catenin–Lef-Tcf pathway in cells expressing the β_2AR-Rfz1 chimera. (**A**) F9 clones treated with β-agonist ISO (10 μM) or β-antagonist PROP (10 μM). Clones were treated with ligand for 4 days and then fixed and stained with TROMA-1 antibody to reveal PE formation. Indirect immunofluorescence (IIF) and phase-contrast (PC) images are displayed. (**B**) Xwnt-8–induced expression of pTOPFLASH. Conditioned medium was collected from clones stably transfected with Xwnt-5a, Xwnt-8, or the empty vector (EV) and used to supplement 1:9 (ratio of conditioned medium of target Rfz-expressing clones to that of the Wnt-expressing clones) the medium of the F9 cells stably expressing either Rfz1, Rfz2, or the empty vector, in tandem with transient cotransfection with pTOPFLASH. (**C**) ISO-induced expression of pTOPFLASH. Clones stably expressing either β_2AR-Rfz1 or β_2AR-Rfz2 chimera were transiently transfected with either pTOPFLASH or pFOPFLASH for 24 hours and then treated with ISO, PROP, or both, for 6 hours, and then activation of the luciferase reporter genes was measured. The data shown in (B) and (C) are the mean values ± SEM of four separate experiments and are reported in relative light units (RLU; 10,000 RLU equals expression of 1 pg of luciferase). * denotes $P < 0.05$ and ** $P < 0.01$ for the difference from unstimulated values (SigmaStat software, SPSS Science).

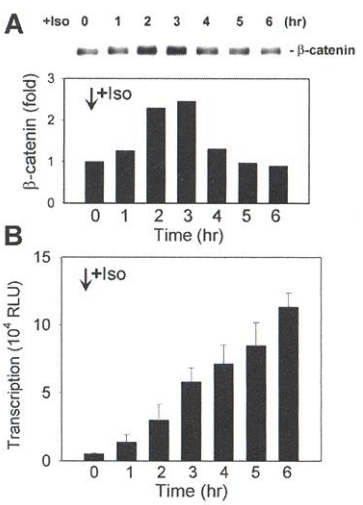

Fig. 3. Activation of β_2AR-Rfz1 chimera in F9 stem cells provokes stabilization of β-catenin and the activation of the Lef-Tcf–sensitive luciferase reporter gene. Activation of β_2AR-Rfz1 chimera in F9 stem cells after clones stably expressing the β_2AR-Rfz1 chimera were treated with ISO at time = 0. Over the next 6 hours, the intracellular concentration of β-catenin and the activation of the Lef-Tcf–sensitive luciferase reporter gene pTOPFLASH were measured. (**A**) The stability of β-catenin was assessed by SDS-PAGE of the intracellular complement of β-catenin. The proteins were transferred to blots, stained with antibodies to β-catenin, and the amount of stain was quantified. (**B**) Transcriptional activation of pTOPFLASH. The data shown are the mean values ± SEM of five separate experiments and are reported in relative light units (RLU).

of pTOPFLASH, but did antagonize the stimulation by ISO. ISO failed to stimulate the pTOPFLASH reported in clones expressing the β_2AR-Rfz2 version of the chimera, further demonstrating the specificity of the Rfz1 chimera activation of the Lef-Tcf pathway.

A hallmark of the Rfz1 pathway is the stabilization of β-catenin in response to Wnt. The time-course for β-catenin stabilization (Fig. 3A) and pTOPFLASH activation (Fig. 3B) was studied in clones expressing β_2AR-Rfz1 (*17*). Stabilization of β-catenin was observed in clones expressing the chimera, within 1 to 2 hours of stimulation with ISO. The stabilization of β-catenin peaked at 3 hours after treatment with ISO, thereafter declining to basal levels within the next 3 hours. ISO stimulated increased activation of the Lef-Tcf–sensitive luciferase reporter gene within the first several hours. Unlike the β-catenin stabilization, the activation of the Lef-Tcf response continued to increase for up to 6 hours, 2 to 3 hours after the β-catenin levels had returned to basal levels.

One of the fundamental questions in understanding how signaling pathways regulate developmental processes is whether or not the activation of β-catenin–Lef-Tcf pathway by *Frizzled* homologs involves heterotrimeric G proteins. It has been reported that Frizzled signaling through the Wnt/Ca++ pathway, that does not involve β-catenin, requires G proteins (*18, 19*), but no data have shown a comparable role for G proteins in the Wnt–β-catenin pathway. The GTP-induced shift in agonist affinity of the β_2AR-Rfz1 chimera (Fig. 1D) suggests that the chimera is a G protein–linked receptor. Pertussis toxin (PT) which ADP-ribosylates and inactivates several subfamilies of heterotrimeric G protein α-subunits, including Gi, Go, and Gt, provided an additional test. Treating β_2AR-Rfz1 chimera-expressing clones with PT for 4 hours reduced the activation of pTOPFLASH by ISO (Fig. 4A). Further elucidation of specific G proteins involved in the Rfz1–β-catenin–Lef-Tcf pathway was performed via suppression of various G protein subunits with antisense oligodeoxynucleotides [ODN (*20, 21*)]. Suppression of either Gαo or Gαq resulted in a suppression of signaling from β_2AR-Rfz1 to the activation of pTOPFLASH. These data reveal an obligate role for specific G proteins in signaling to the β-catenin–Lef-Tcf pathway. The overall pathway is sensitive to PT treatment and suppression of either Gαo (a PT substrate) or Gαq precludes the activation of the pathway, as highlighted in a 24-hour time-course for ISO-stimulated activation of pTOPFLASH transcription in clones expressing the β_2AR-Rfz1 chimera (Fig. 4C).

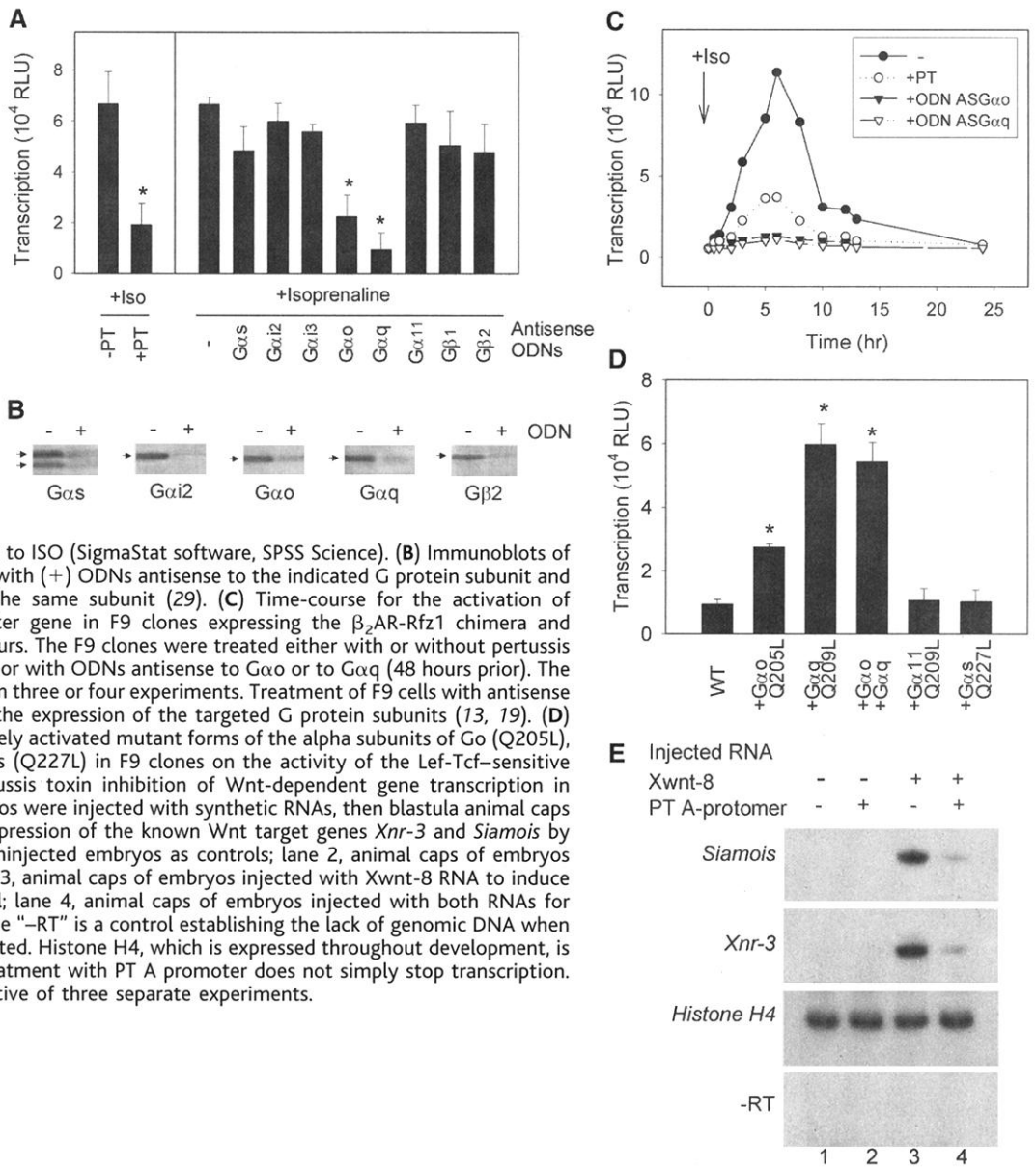

Fig. 4. Activation of the β-catenin–Lef-Tcf–sensitive pathway: effects of PT and suppression of G protein subunits. Sensitivity of the ISO-stimulated pTOPFLASH activation to pretreatment with PT and ODNs antisense to G protein subunits. Clones expressing the β_2AR-Rfz1 chimera were treated either for 4 hours with PT (10 ng/ml) or for 48 to 72 hours with antisense ODNs (**A**) and sense/missense (*24*) ODNs to suppress specific G protein subunits. Clones were then treated with ISO for an additional 6 hours, and the pTOPFLASH activation was measured. The data are the mean values of triplicate determinations of a representative experiment and are reported in relative light units (RLU). * denotes $P < 0.05$ for the difference from the cells not exposed to ISO (SigmaStat software, SPSS Science). (**B**) Immunoblots of F9 clones treated without (–) and with (+) ODNs antisense to the indicated G protein subunit and stained with antibodies against the same subunit (*29*). (**C**) Time-course for the activation of Lef-Tcf–sensitive luciferase reporter gene in F9 clones expressing the β_2AR-Rfz1 chimera and treated with 10 μM ISO for 24 hours. The F9 clones were treated either with or without pertussis toxin (PT, 10 ng/ml, 4 hours prior) or with ODNs antisense to Gαo or to Gαq (48 hours prior). The results shown are mean values from three or four experiments. Treatment of F9 cells with antisense ODNs provokes a suppression of the expression of the targeted G protein subunits (*13, 19*). (**D**) Effects of expression of constitutively activated mutant forms of the alpha subunits of Go (Q205L), Gq (Q209L), G11 (Q209L), and Gs (Q227L) in F9 clones on the activity of the Lef-Tcf–sensitive luciferase reporter gene. (**E**) Pertussis toxin inhibition of Wnt-dependent gene transcription in *Xenopus* embryos. *Xenopus* embryos were injected with synthetic RNAs, then blastula animal caps were isolated and analyzed for expression of the known Wnt target genes *Xnr-3* and *Siamois* by RT-PCR. Lane 1, animal caps of uninjected embryos as controls; lane 2, animal caps of embryos injected with PT A protomer; lane 3, animal caps of embryos injected with Xwnt-8 RNA to induce target genes, as a positive control; lane 4, animal caps of embryos injected with both RNAs for Xwnt-8 and the PT A protomer. The "–RT" is a control establishing the lack of genomic DNA when the reverse transcriptase was omitted. Histone H4, which is expressed throughout development, is a control establishing that the treatment with PT A promoter does not simply stop transcription. These experiments are representative of three separate experiments.

Thus, the β_2AR-Rfz1 chimeric receptor displays an agonist-specific, GTP-dependent shift in receptor affinity (Fig. 1D) and a response sensitive both to PT and to suppression of Gαo and Gαq (Fig. 4A), properties characteristic of members of the superfamily of G protein–linked receptors. Results from the expression of constitutively active, GTPase forms of Gαo (Q205L) and of Gαq (Q209L) show that expression of Q209L Gαq and, to a lesser extent, expression of Q205L Gαo constitutively activate the Lef-Tcf pathway (Fig. 4D). Expression of constitutively active versions of Gα11 and Gαs, in constrast, had no effect on pTOPFLASH activity.

Our results above implicate PT-sensitive G proteins as playing a direct role in the activation of β-catenin target genes. However, Wu *et al.* (*22*) have recently reported that PT does not block the ability of Xwnt-8 to induce an ectopic axis in *Xenopus* embryos, a process that relies on the expression of β-catenin target genes. Given the apparent discrepancy, we directly tested whether the ability of Xwnt-8 to induce expression of two known direct targets of β-catenin, *Siamois* and *Xnr-3* (*23*), could be blocked by pertussis toxin (Fig. 4E). We confirmed that blastula animal cap explants do not express either gene (Fig. 4E), and that PT does not provoke expression of either gene by itself. Expression of Xwnt-8 in explants caused increased transcription of both *Siamois* and *Xnr-3*. Injection of RNA encoding the A protomer of pertussis toxin before the injection of Xwnt-8 RNA, however, attenuated the activation of both of the target genes. Similarly, expression of EF1-alpha was equivalent in all four lanes (*24*). These data demonstrate that the PT treatment specifically inhibits transcription of β-catenin target genes. The data, measuring activation of endogenous direct targets of β-catenin signaling, confirm our conclusions in F9 cells that pertussis toxin attenuates activation of β-catenin target genes.

The classical Wnt–β-catenin signaling paradigm is based on the ability of frizzled signals to enhance the cellular accumulation of β-catenin in the nucleus and to activate the Lef-Tcf transcriptional complex (*1, 5, 6*). What has not been evident is whether there is a requirement for specific G protein subunits early in the response, before stabilization of β-catenin. Our studies with a chimeric receptor show that it binds agonist and provokes formation of PE. Activation of the chimera provokes stabilization of β-catenin and rapid transcription of a β-catenin–Lef-Tcf reporter gene. These effects are sensitive to PT as well as depletion of Gαo and Gαq. The ability of the cytoplasmic domains of the Rfz1 to enable the chimera to signal to the β-catenin–Lef-Tcf pathway and to enable an agonist-specific GTP-dependent shift of chimera receptor affinity argue that the *Frizzled* gene product is a G protein–linked receptor. Further evidence in support of our proposal is the recently reported ability of RGS proteins to inhibit Xwnt-8 signaling in *Xenopus* (*22*). Our observations suggest that the activation of the classical β-catenin–Lef-Tcf pathway in mammalian cells is G protein–linked and that Rfz1 is a G protein–linked receptor.

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8. A β_2AR-Rfz1 chimera was created and inserted into an expression vector. Primers were synthesized by Operon. The sequences are as follows: FZ1: GAAGATCTGCGATGGGGCCACCCGGG; FZ2: GTAGCTGAATCGCCGCATGTCAATGGC; FZ3: CATTGACATGCGGCGATTCAGCTACCC; FZ4: TCGTGGCCCCACTTCATATCCACTGCTATCACG; FZ5: AGTGGATATGAAGTGGGGCCACGAGGCCATCG; FZ6: CACCATGAGCTTCTCCAGCTTCTCCGTCTTGG; FZ7: GAGAAGCTGGAGAAGCTCATGGTGTTAGGCATCATCATGGG; FZ8: CCTCCACGAGTTCAGCGTCTTGC; FZ9: GGCAAGACGCTGAACTCGTGGAGG; FZ10: GCTCTAGATCACACGGTGGTCTCTCCTTGCTTGCTGTTGGTGAGACGC. Step 1: To construct the β_2AR-Rfz1 chimera, pCDNA3β_2AR-Rfz2 plasmid was used as a template, and five PCR fragments were obtained (fragments 1, 2, 3, 4, and 5) when oligos FZ 1 and FZ 2; FZ 3 and FZ 4; FZ 5 and FZ 6; FZ 7 and FZ 8; and FZ 9 and FZ 10 were used as pairs for primers, respectively. Step 2: Using fragments 2 and 3 as a template, fragment 2/3 was obtained when oligos FZ 3 and FZ 6 were employed as primers. Similarly, using fragments 3 and 4 as a template, fragment 3/4 was obtained when oligos FZ 5 and FZ 8 were employed as primers. Step 3: Using fragments 1 and 2/3 as a template, fragment 1/23 was obtained when oligos FZ 1 and FZ 6 were employed as primers. Using fragments 3/4 and 5 as a template, fragment 3/4/5 was obtained when oligos FZ 5 and FZ 10 were employed as primers. Step 4: Fragment 1/2/3 was digested with Eco RI and Bam HI and then inserted into the Eco RI and Bam HI site of pBluescript SK (Stratagene), Fragment 3/4/5 was digested with Bam HI and XbaI and inserted into the Bam HI and XbaI site of the above recombinant plasmid, pBSKβ_2AR/Fz1. The plasmid was cut with Eco RI and Not I, and the small fragment was inserted into the Eco RI-Not I site of pCDNA3. The recombinant is pCDNA3β_2AR/Fz1. For studies in *Xenopus* animal caps, the plasmid was digested with Eco RI and XbaI, and the small fragment was inserted into the Eco RI–XbaI site of pCS2+. The recombinant plasmid employed in the studies in *Xenopus* is termed pCS2+β_2AR/Fz1.
9. The mouse F9 teratocarcinoma cells were obtained from the ATCC collection, propagated, and stably transfected using Lipofectamine (Gibco-BRL, Life Technologies). The cells were transfected with pCDNA3 expression vector (Invitrogen) alone (empty vector) or pCDNA3 vector engineered by standard techniques to express either Xwnt-5a, Xwnt-8, rat Frizzled-1, rat Frizzled-2, the constitutively activated mutant forms of one of the α-subunits of Go (Q205L), Gq (Q209L), G11 (Q209L), and Gs (Q227L), or either the β_2AR-Rfz1 or β_2AR-Rfz2 chimera under the control of the cytomegalovirus promoter (*13*). Some 10 to 20 independent clones that were resistant to G418 were propagated in each transfection. The expression of the mRNA for each of the target proteins was measured indirectly via RT-PCR. The clones displaying the highest level of expression of mRNA for Xwnt-5a (*25*), Xwnt-8 (*26*), rat Frizzled-1 or -2 (Rfz1 or Rfz2) (*27*), or the chimera were employed for the studies.
10. H. Y. Wang *et al.*, unpublished data.
11. Aliquots of crude membrane fractions (0.2 mg protein per lane) from each F9 clone were subjected to either radioligand binding analysis by standard procedures (*28*) or SDS–polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins transferred to nitrocellulose, and blots were stained with a rabbit polyclonal, anti-peptide antibodies to the exofacial epitope of the β_2-adrenergic receptor (CM04). The immune complexes were made visible by staining with a second antibody (goat anti-rabbit immunoglobulin G) coupled to calf alkaline phosphatase (*29*).
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14. Clones expressing the β_2AR-Rfz1 chimera were propagated and then treated with or without the β-agonist isoproterenol, the β-antagonist propranolol, or both agents, in combination. At the end of 4 days, the clones were fixed with 3% paraformaldehyde for 5 min, stained with the PE-specific marker antigen cytokeratin endo-A by the monoclonal antibody TROMA-1 (University of Iowa Developmental Studies Hybridoma Bank, Iowa City, IA).
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16. F9 clones were stably transfected to express the β_2AR-Rfz1 chimera and then transiently transfected either with the pTOPFLASH plasmid, which contains three copies of the optimal Tcf motif CCTTTGATC upstream of a minimal c-Fos promoter driving luciferase expression or the pFOPFLASH plasmid, which contains a mutated motif CCTTTGGCC of the Lef-Tcf–sensitive elements (*15*). The clones were cotransfected with a second plasmid that expresses the gene for β-galactosidase, which was used to measure the efficiency of the transfection. Clones were treated with 10 μM isoproterenol, and the activation of Lef-Tcf–sensitive luciferase reporter gene was determined.
17. For study of β-catenin stabilization, clones stably expressing β_2AR-Rfz1 chimera were treated with 10 μM isoproterenol, and β-catenin levels were determined in supernatants of whole-cell lysates treated with ConA-Sepharose to remove cadherin-associated β-catenin. Portions of the cell supernatants were subjected to SDS-PAGE, and the resolved protein was transferred to blots. Immunoblots of β-catenin were stained with antibodies to β-catenin (Sigma #C2206) and detected by enhanced chemiluminescence, and the amount of stain was quantified by imaging densitometry on a Bio-Rad GS-700.
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21. The F9 clones expressing the β_2AR-Rfz1 receptor were propagated on 12- or 24-well plates (2000 to 5000 cells/well) and allowed to attach overnight. The clones were treated with phosphorothioate oligodeoxynucleotides (cell culture–grade, HPLC-purified ODNs from Operon Technologies) antisense to specific G protein subunits at least 48 hours in advance of challenge with 10 μM isoproterenol (*20*).
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23. *Xenopus* embryos were injected with RNA encoding either pertussis toxin A protomer (4 ng at the four-cell stage), or RNA encoding Xwnt-8 [10 pg mixed with 4 ng of control RNA (GFP) at the 16-cell stage], or RNA encoding both Xwnt-8 (10 pg) and pertussis toxin A protomer (4 ng). The embryos were cultured to embryonic stage 8 (6 to 7 hours after fertilization), then animal cap explants were removed

and cultured to stage 10 (2 to 3 hours). Twenty explants were processed in each sample and the presence of mRNA encoding the Wnt-responsive genes *Siamois* and *Xnr-3* and control (Histone H4 and translational elongation factor 1α, *EF-1α*) was detected by RT-PCR analysis at the 16-cell stage, as described elsewhere (3).

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A Transgenic Model for Listeriosis: Role of Internalin in Crossing the Intestinal Barrier

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Listeria monocytogenes is responsible for severe food-borne infections, but the mechanisms by which bacteria cross the intestinal barrier are unknown. *Listeria monocytogenes* expresses a surface protein, internalin, that interacts with a host receptor, E-cadherin, to promote entry into human epithelial cells. Murine E-cadherin, in contrast to guinea pig E-cadherin, does not interact with internalin, excluding the mouse as a model for addressing internalin function in vivo. In guinea pigs and transgenic mice expressing human E-cadherin, internalin was found to mediate invasion of enterocytes and crossing of the intestinal barrier. These results illustrate how relevant animal models for human infections can be generated.

Understanding how bacteria cross the intestinal barrier is a key issue in the study of foodborne diseases. *Listeria monocytogenes* causes listeriosis, an infection characterized by bacterial dissemination from the intestinal lumen to the central nervous system and the fetoplacental unit (*1*). As recently shown, *L. monocytogenes* is also responsible for gastroenteritis (*2*, *3*). How this bacterium crosses the intestinal barrier is unknown. In vitro, the *L. monocytogenes* surface protein internalin promotes bacterial internalization into human epithelial cells (*4*). Its receptor is E-cadherin (*5*), a protein that mediates the formation of adherens junctions between epithelial cells and is also expressed on the basolateral face of polarized epithelial cells (*6*). In contrast to human E-cadherin (hEcad), mouse and rat E-cadherins are not receptors for internalin, and internalin plays no role in entry into mouse and rat epithelial cells (*7*). We have shown that this specificity relies on the nature of the sixteenth amino acid, a proline in hEcad, and a glutamic acid in mouse and rat E-cadherins (*7*). We concluded that although rat and mouse can be successfully used to study the T cell response to intravenous (IV) infection of *L. monocytogenes*, they are inappropriate models for studying internalin function in vivo. In mice, oral infections are not reproducibly lethal, and bacterial translocation across the intestinal barrier is low. Moreover, in these animals, specific targeting to the brainstem and the fetoplacental unit is not seen, even after IV inoculation.

Guided by the pioneering work of Racz *et al.* (*8*), we observed that a guinea pig epithelial cell line allows entry of *L. monocytogenes* strain EGD at an efficiency 200 times that of isogenic internalin deletion mutant EGDΔ*inlA* (*7*). These results correlated with the finding that guinea pig E-cadherin harbors a proline at position 16 and led us to propose that the role of internalin in vivo could be addressed with guinea pigs or transgenic mice expressing hEcad. We report the results of oral infections of both guinea pigs and transgenic mice expressing hEcad on their enterocytes and show that internalin is a virulence factor mediating crossing of the intestinal barrier.

IV infections of mice with *L. monocytogenes* are lethal. The median lethal dose (LD_{50}) of EGD or EGDΔ*inlA* is similar ($\sim 10^5$ bacteria) (*9*). In contrast, oral infections with even 5×10^{10} EGD or EGDΔ*inlA*

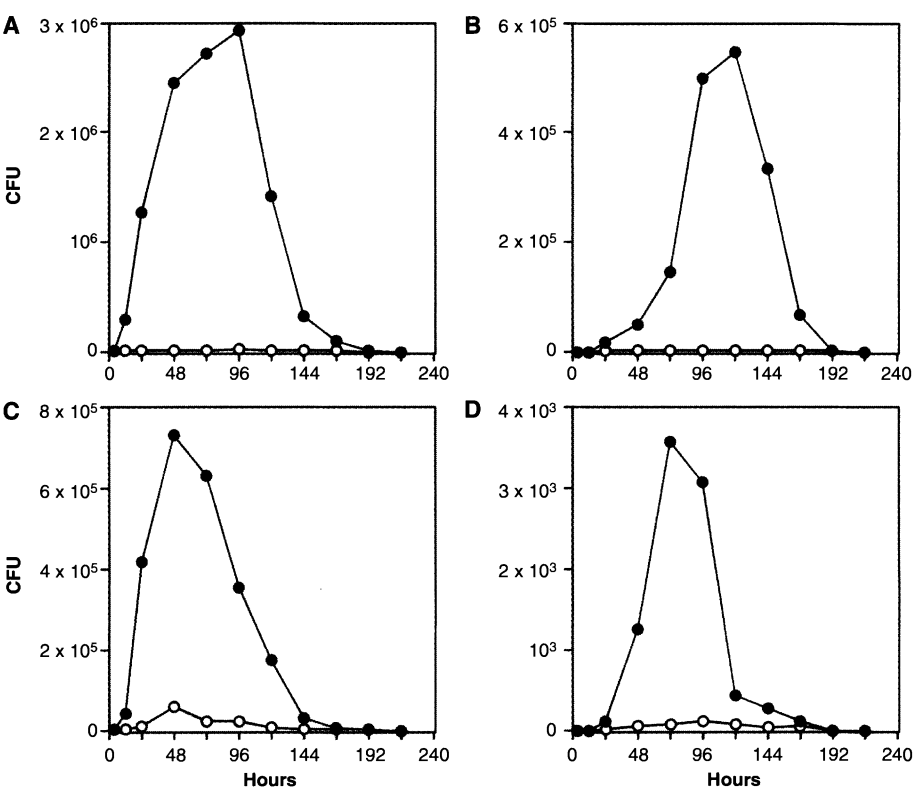

Fig. 1. Role of internalin in the bacterial invasion of guinea pig (**A**) small intestine, (**B**) liver, (**C**) mesenteric lymph nodes, and (**D**) spleen. Bacterial counts in organs of guinea pigs infected orally with 10^{10} *L. monocytogenes* wild-type strain EGD (purple plots) and the internalin deletion mutant EGDΔ*inlA* (white plots) are shown (9). Mean colony-forming units (CFU) from four different animals are given at each time point.

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