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Membrane-anchored Plakoglobins Have Multiple Mechanisms of Action in Wnt Signaling

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In Wnt signaling, β-catenin and plakoglobin transduce signals to the nucleus through interactions with TCF-type transcription factors. However, when plakoglobin is artificially engineered to restrict it to the cytoplasm by fusion with the transmembrane domain of connexin (cnxPg), it efficiently induces a Wnt-like axis duplication phenotype in *Xenopus*. In *Xenopus* embryos, maternal XTCF3 normally represses ventral expression of the dorsalizing gene *Siamois*. Two models have been proposed to explain the Wnt-like activity of cnxPg: 1) that cnxPg inhibits the machinery involved in the turnover of cytosolic β-catenin, which then accumulates and inhibits maternal XTCF3, and 2) that cnxPg directly acts to inhibit XTCF3 activity. To distinguish between these models, we created a series of N-terminal deletion mutations of cnxPg and examined their ability to induce an ectopic axis in *Xenopus*, activate a TCF-responsive reporter (OT), stabilize β-catenin, and colocalize with components of the Wnt signaling pathway. cnxPg does not colocalize with the Wnt pathway component Dishevelled, but it does lead to the redistribution of APC and Axin, two proteins involved in the regulation of β-catenin turnover. Expression of cnxPg increases levels of cytosolic β-catenin; however, this effect does not completely explain its signaling activity. Although cnxPg and Wnt-1 stabilize β-catenin to similar extents, cnxPg activates OT to 10- to 20-fold higher levels than Wnt-1. Moreover, although LEF1 and TCF4 synergize with β-catenin and plakoglobin to activate OT, both suppress the signaling activity of cnxPg. In contrast, XTCF3 suppresses the signaling activity of both β-catenin and cnxPg. Both exogenous XLEF1 and XTCF3 are sequestered in the cytoplasm of *Xenopus* cells by cnxPg. Based on these data, we conclude that, in addition to its effects on β-catenin, cnxPg interacts with other components of the Wnt pathway, perhaps TCFs, and that these interactions contribute to its signaling activity.

INTRODUCTION

Wnts are secreted glycoproteins that are thought to act locally (see Nusse and Varmus, 1992; Cadigan and Nusse, 1997). A combination of genetic, biochemical, and cell biological studies have led to a consensus model for the Wnt signaling pathway. Binding of a secreted Wnt to Frizzled/Frizzled-2–type receptors (Bhanot *et al.*, 1996; Bhat, 1998; Kennerdell and Carthew, 1998; Muller *et al.*, 1999) activates the protein Dishevelled (Dvl) (Yanagawa *et al.*, 1995), which in turn leads to the inactivation of glycogen synthase kinase-3β (Gsk3β). Gsk3β, a ubiquitous enzyme (see Yost *et al.*, 1997), regulates levels of β-catenin (Siegfried *et al.*, 1992) by phosphorylating serine and threonine residues in the N terminus of cytosolic β-catenin (Yost *et al.*, 1996) and targeting β-catenin for proteolytic degradation. Thus, in response to a Wnt signal, Gsk3β activity is inhibited and cytosolic β-catenin accumulates and is available to interact with TCF-type transcription factors (Behrens *et al.*, 1996; Huber *et al.*, 1996; Molenaar *et al.*, 1996; Brunner *et al.*, 1997; van de Wetering *et al.*, 1997), which alter the expression of target genes.

The proteolytic degradation of cytosolic β-catenin and its closely related vertebrate paralogue plakoglobin/γ-catenin appears to involve the product of the *adenomatous polyposis coli* (*APC*) gene (Munemitsu *et al.*, 1995). β-Catenin and

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Abbreviations used: APC, adenomatous polyposis coli; cnxPg, connexin-plakoglobin; Dvl, dishevelled; GFP, green fluorescent protein; Gsk3β, glycogen synthase kinase 3β; mt, myc tag; TBS, Tris-buffered saline; TCFs, LEF/TCF proteins.

plakoglobin form complexes with APC and Gsk3β (Rubinfeld *et al.*, 1993, 1995, 1996; Hulsken *et al.*, 1994) to target these proteins for ubiquitination and degradation via the proteosome (Aberle *et al.*, 1997). Axin and the related protein Conductin/Axil, which negatively regulate the Wnt pathway, link Gsk3β, β-catenin, and APC into a single complex (Yamamoto *et al.*, 1988; Zeng *et al.*, 1997; Behrens *et al.*, 1998; Ikeda *et al.*, 1998; Kishida *et al.*, 1998; Sakanaka *et al.*, 1998). Mutations in *APC*, responsible for most cases of familial colon cancer, act, at least in part, by increasing the stability of β-catenin (Korinek *et al.*, 1997). A substantial number of colon cancers without apparent mutations in *APC* have mutations in *β-catenin* that stabilize the cytosolic form of the protein (Morin *et al.*, 1997; Sparks *et al.*, 1998). Similar mutations in *β-catenin* have been found in cultured melanoma cell lines (Rubinfeld *et al.*, 1997), medulloblastomas (Zurawel *et al.*, 1998), pilocytomas (Chan *et al.*, 1999), and carcinomas of the liver (de La Coste *et al.*, 1998; Miyoshi *et al.*, 1998), prostate (Voeller *et al.*, 1998), and endometrium (Fukuchi *et al.*, 1998; Palacios and Gamallo, 1998).

In addition to its role in Wnt signaling, β-catenin plays a critical role in cadherin-mediated cell-cell adhesion, in which it links the classic cadherins of adherens junctions to actin filaments through α-catenin (see Yap *et al.*, 1997). Plakoglobin has a similar role at the adherens junction, and expression of either vertebrate plakoglobin or β-catenin in *Drosophila* can rescue the adhesion defects associated with *armadillo* mutants (White *et al.*, 1998). β-Catenin and plakoglobin have also been found to be associated with the tight junction protein ZO-1 (Rajasekaran *et al.*, 1996), the EGF receptor (Hoschuetzky *et al.*, 1994; Kanai *et al.*, 1995), tyrosine phosphatases (Kypta *et al.*, 1996), the actin-bundling protein fascin (Tao *et al.*, 1996), the integral membrane protein Presenilin-1 (Zhou *et al.*, 1997; Murayama *et al.*, 1998; Yu *et al.*, 1998), the TATA box–binding protein pontin-52 (Bauer *et al.*, 1998), SOX-type transcription factors (Zorn *et al.*, 1999) and APC, Axin, and Conductin/Axil (see above). β-Catenin and plakoglobin differ most dramatically in the ability of plakoglobin, but not β-catenin, to bind to desmosomal cadherins (see Gelderloos *et al.*, 1997; however, also see Bierkamp *et al.*, 1999). Differences in their interactions with adhesion complexes have been observed in vivo (see Nathke *et al.*, 1994; Lampugnani *et al.*, 1995). In particular, tissues, β-catenin, and plakoglobin can be present at different levels and in different complexes (see Simcha *et al.*, 1998).

The HMG-type transcription factors known as lymphoid enhancer factor-1 (LEF1) and T-cell enhancer factors (TCFs), which we refer to generically as TCFs, mediate the final steps in Wnt signaling through their interactions with β-catenin and plakoglobin. The prevailing model for Wnt signaling states that in response to a Wnt stimulus, hypophosphorylated forms of β-catenin or plakoglobin accumulate and form complexes with TCFs. These heterodimeric complexes are then thought to activate transcription at TCF target gene promoters. Alternatively, several lines of evidence suggest that certain TCFs may act as constitutive repressors at target promoters, whose repression is relieved by binding to β-catenin or plakoglobin (see Klymkowsky, 1997, and below). In *Drosophila*, mutation of the TCF-binding site in the *Ultrabithorax* midgut enhancer augments expression of *Ubx* in cells not exposed to the Wingless protein (Riese *et al.*, 1997). A similar derepression is observed upon mutation of TCF sites in the *Siamois* enhancer, increasing ventral production of *Siamois*; such mutations have no effect on dorsal expression (Brannon *et al.*, 1997). More recent work has solidified the notion of TCFs as transcriptional repressors (see Bienz, 1998), e.g., Groucho family members can bind TCFs and mediate constitutive transcriptional repression at TCF-binding sites (Roose *et al.*, 1998; see also Cavallo *et al.*, 1998; Levanon *et al.*, 1998).

We originally constructed membrane-anchored forms of plakoglobin to study whether plakoglobin's nuclear localization was required to induce axis duplication in *Xenopus*. When RNAs encoding these polypeptides were injected into fertilized *Xenopus* eggs, they were effective at inducing axis duplication and did not appear to alter nuclear β-catenin levels (Merriam *et al.*, 1997). Based on this result and the inhibitory effects of mutated XTCF3 on axis formation (Molenaar *et al.*, 1996), we argued that plakoglobin (and β-catenin) inhibited the repressive activity of maternal XTCF3, allowing the ventral expression of *Siamois* and other dorsalizing genes (see Klymkowsky, 1997; Merriam *et al.*, 1997). Subsequently, Miller and Moon (1997) and Hsu *et al.* (1998) reported that analogous anchored forms of β-catenin inhibit β-catenin degradation and thereby stabilized endogenous β-catenin, arguing that the effects of "anchored catenins" are indirect and dependent on endogenous β-catenin. Most recently, Cox *et al.* (1999) reported that a "membrane-tethered" form of armadillo did not modulate gene expression in the absence of wild-type armadillo, arguing for an indirect mode of action.

To resolve these apparent discrepancies, we examined further the signaling activities of membrane-anchored plakoglobins using a panel of deletion mutants. Our studies in human 293T and *Xenopus* A6 cell lines confirm that anchored plakoglobins do act to increase levels of cytosolic β-catenin. However, several lines of evidence indicate that the signaling activity of anchored plakoglobin is not simply due to its effects on cytosolic β-catenin. We show that XTCF3 acts negatively, suppressing the ability of β-catenin to activate a TCF-responsive promoter, and that connexin-plakoglobins (cnxPgs) can sequester TCF family members in the cytoplasm. The results obtained with these admittedly artificial polypeptides raise the intriguing possibility that cytoplasmic forms of catenins may modulate the nuclear availability of TCFs and other negatively acting factors.

MATERIALS AND METHODS

Plasmids

For expression of proteins in *Xenopus* A6 and human 293T cells, we used the pCS2mt plasmid developed by Rupp and Turner (Rupp *et al.*, 1994) or the pCDNA3 plasmid (Invitrogen, Carlsbad, CA). For synthesis of cnxPg RNAs for embryo injection, we used the pT7 plasmid described previously (Karnovsky and Klymkowsky, 1995; Merriam *et al.*, 1997). The pT7cnx-human plakoglobin-myc (pT7cnxPg-myc), pT7cnx-human plakoglobin-green fluorescent protein (pT7cnxPg-GFP), pT7N2ΔPg-myc, and pT7N5ΔPg-myc plasmids have been described previously (Merriam *et al.*, 1997; Rubenstein *et al.*, 1997). The GFP in these plasmids contains a S65→T mutation that enhances its fluorescence (Heim *et al.*, 1995). The N2ΔPg sequence was subcloned into pCS2mt-GFP using *Eco*RI and *Xba*I sites to form pCS2mt-N2ΔPg-GFP. To subclone cnxPg into pCS2 plasmids, pT7cnxPg-myc was digested with *Hin*dIII and *Xba*I and the released fragment was subcloned into pCS2mt-GFP. The

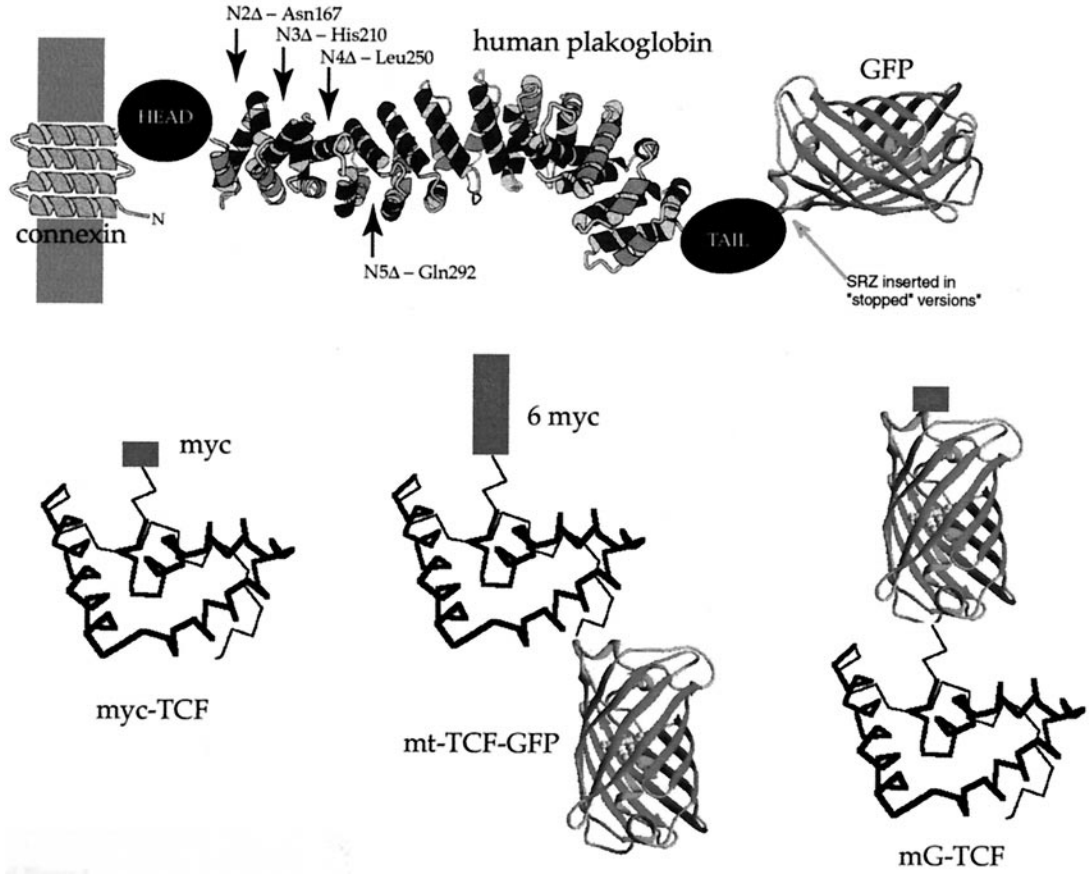

Figure 1. Constructs. (Top) Cartoon of cnxPg, based on the crystal structure of the closely related protein β-catenin (Huber *et al.*, 1997), that illustrates the cnxPg constructs used in this work. The positions of the first plakoglobin-derived amino acid in the various deletions are indicated. N2Δ fuses the connexin transmembrane domain to Asn-167 of plakoglobin, N3Δ fuses the connexin domain to His-210 of plakoglobin, N4Δ fuses the connexin domain to Leu-250 of plakoglobin, and N5Δ fuses the connexin domain to Gln-292 of plakoglobin. Each deletion removes the bulk of the N-terminal Arm repeat, leaving five to eight amino acids upstream of the start of the next Arm repeat, as defined structurally. (Bottom) myc- and GFP-tagged forms of TCFs used in this work.

resulting plasmid has the original SP6 RNA polymerase promoter, 5′ untranslated region, and 6-myc tag region of the pCS2mt plasmid replaced with the T7 RNA polymerase promoter and 5′ β-globin untranslated region of the pT7 plasmid. We refer to these plasmids as pCsCnxPg-GFP. PCR was used to amplify the N3ΔPg and N4ΔPg sequences (5′ N3Δ oligonucleotide CCCGAATTCgcacaacctctcccac-cac and 5′ N4Δ oligonucleotide CCCGAATTCgctgcacaacctgctcctg, together with the 3′ full-length human plakoglobin primer CCCCTCTAGAggccagcatgtggtctgc). To create untagged forms of the cnxNΔ2Pg, cnxN3ΔPg, cnxN4ΔPg, and cnxNΔ5Pg polypeptides, the pCsCnx plasmids were digested with *Xba*I and the DNA ends filled in T4 DNA polymerase and religated, creating an in-frame stop codon between the plakoglobin and GFP sequences. Because of the presence of a second, downstream T7 RNA polymerase promoter in the pCsCnx plasmids, we subcloned the cnxN3ΔPg and cnxN4ΔPg sequences from pCsCnx into the pT7-GFP plasmid using *Hin*dIII and *Xba*I to form pT7cnxN3ΔPg-GFP and pT7cnxN4ΔPg-GFP, which were used for in vitro RNA synthesis (see below).

Plasmids encoding an single HA-epitope–tagged form of *Xenopus* TCF3 (HA-XTCF3), a single myc-tagged *Xenopus* LEF-1 (myc-XLEF-1) (Molenaar *et al.*, 1996, 1998), human TCF4 (Korinek *et al.*, 1997), mouse and human LEF1 (Travis *et al.*, 1991; Waterman *et al.*, 1991), a myc-tagged mouse Axin (mtAxin) (Zeng *et al.*, 1997), *Xenopus* Notch (Xotch) (Coffman *et al.*, 1990), and a myc-tagged form of *Xenopus* Dishevelled (mtXDvl) (Sokol, 1996) were generously supplied by Hans Clevers and Miranda Molenaar (University of Nijmegen, Nijmegen, The Netherlands), Rudolf Grosschedl (University of California, San Francisco, CA), Katherine Jones (Salk Institute, La Jolla, CA), Michael Sargent (National Institute for Medical Research, Mill Hill, United Kingdom), Frank Costantini (Columbia University, New York, NY), Clark Coffman (University of Colorado, Boulder, CO), and Sergei Sokol (Harvard Medical School, Cambridge, MA), respectively. A myc-GFP–tagged form of the *Xenopus* zinc-finger transcription factor XSlug has been described elsewhere (Carl *et al.*, 1999). The full nucleotide coding regions of XTCF3 and hLEF1 were amplified using PCR (oligonucleotides for XTCF3: 5′-CCCGAAT-TCGcctcaactaaacagcggcg and 3′-CCCTCTAGAgtcactggatttggtcacc; oligonucleotides for hLEF1: 5′-CCCGAATTCGccccaactttccggagg and 3′-CCCCTCTAGAtcagatgtaggcagctgtcattc). In the case of Xotch, we isolated the cytoplasmic tail domain (5′-CCCGAATTC-caagaagcgtcgccgtgaac and 3′-CCCTCTAGAttacttgaaagcttcagg). The amplified DNAs were digested with *Eco*RI and *Xba*I and were subcloned into pCS2mt-GFP (pCS2mtXTCF3-GFP) and pCS2mycGFP (pCS2mGXTCF3, pCS2mGLEF1, and pCS2mGXotchTail) (Figure 1). In addition, a form of XTCF3 missing the N-terminal 166 amino acids (pCS2mt-ΔN166-XTCF3-GFP) was generated in a similar manner (oli-

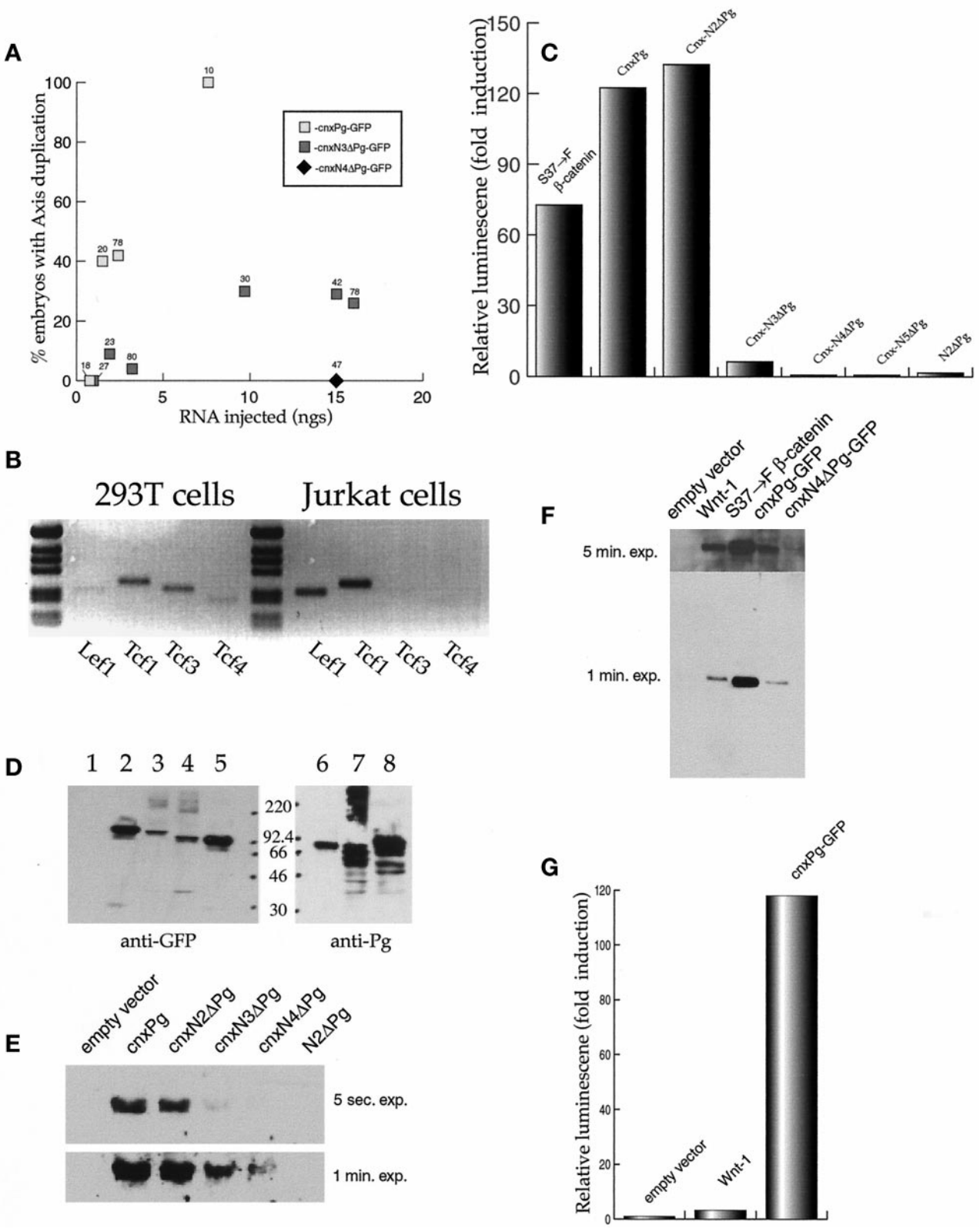
A
% embryos with Axis duplication
RNA injected (ngs)
-cnxPg-GFP
-cnxN3ΔPg-GFP
-cnxN4ΔPg-GFP
B
293T cells
Jurkat cells
Lef1
Tcf1
Tcf3
Tcf4
C
Relative luminescene (fold induction)
S37→F β-catenin
CnxPg
Cnx-N2ΔPg
Cnx-N3ΔPg
Cnx-N4ΔPg
Cnx-N5ΔPg
N2ΔPg
D
anti-GFP
anti-Pg
E
empty vector
cnxPg
cnxN2ΔPg
cnxN3ΔPg
cnxN4ΔPg
N2ΔPg
5 sec. exp.
1 min. exp.
F
empty vector
Wnt-1
S37→F β-catenin
cnxPg-GFP
cnxN4ΔPg-GFP
5 min. exp.
1 min. exp.
G
Relative luminescene (fold induction)
empty vector
Wnt-1
cnxPg-GFP

Figure 2.

gonucleotides 5′ CCCGAATTCCcacccacttacgcctctcatcacc and the 3′ XTCF3 above). Plasmids expressing an S37→F mutated form of human β-catenin were obtained from Paul Robbins (Surgery Branch, National Cancer Institute, Bethesda, MD). The coding sequence was amplified and subcloned into pCDNA3. Sequence analysis revealed the presence of an additional point mutation in this construct (D32→V). We have subsequently shown that a version of β-catenin containing only a mutation in S37 (S37→A) acts identically in all assays to the D32→V/S37→F (referred to as S37→F) mutant described here (our unpublished results). We have also used the stabilized "pt-mutant" form of *Xenopus* β-catenin, which was described by Yost *et al.* (1996) and supplied to us by Aaron Zorn (Wellcome/CRC Institute, Cambridge, United Kingdom). A mutated version of human plakoglobin (S28→A), derived from a human plakoglobin cDNA (Franke *et al.*, 1989), was also constructed. The wild-type plasmid was mutagenized using the Stratagene (La Jolla, CA) QuikChange mutagenesis system with primers that placed a silent *Ngo*MI site in the "mutator" oligonucleotide together with the addition of a S→A mutation in the 28th residue. Sequencing confirmed the presence of the expected mutation.

Figure 2 (facing page) The axis-inducing activities of the cnxPg-GFPs. (A) Fertilized *Xenopus* eggs, injected with various amounts of capped RNA (20 nl injected), were allowed to develop to stages 15–18 and then assayed for dorsal axis duplication. Only embryos that were fluorescent as a result of the expression of exogenous protein were counted; the numbers above each point indicate the total number of embryos scored. (B) Expression of TCFs in 293T cells. Reverse transcriptase-PCR analysis was used to analyze TCF expression. Human Jurkat cells express TCF1 and LEF1 RNAs. 293T cells express detectable levels of all four TCF RNAs, i.e., TCF1, LEF1, TCF3, and TCF4. Reactions in which ribonuclease was added showed no DNA amplification (our unpublished results). (C) cnxPg induction of the OT reporter. Human 293T cells were transfected with plasmids (2 μg of DNA) that express S37→F–β-catenin, N2ΔPg, or various forms of cnxPg, together with the OT reporter and a plasmid driving the expression of β-galactosidase. Luciferase activity was measured after 36 h and was normalized to β-galactosidase activity levels. All transfections used a total of 4.0 μg of DNA. Activity of the "empty vector" was normalized to 1. In each case, the pattern of reporter activation shown was reproduced in at least three independent experiments. (D) Accumulation of cnxPgs. 293T cells were transfected with 4 μg of plasmid DNA; 36 h later, the cells were homogenized and extracts were analyzed by immunoblot (lanes 1 and 6, empty vector; lane 2, cnxPg-GFP; lane 3, cnxN2ΔPg-GFP; lane 4, cnxN4ΔPg-GFP; lanes 5 and 8, cnxN5ΔPg-GFP; lane 7, cnxN3ΔPg) using either an anti-GFP antibody (lanes 1–5) or an anti-plakoglobin antibody (lanes 6–8). The cnxPg polypeptides consistently run with an apparent molecular weight lower than their calculated size. The positions of molecular weight markers are noted. (E) Stabilization of soluble β-catenin by cnxPgs. 293T cells were transfected with plasmids (2 μg of DNA) driving the expression of various cnxPgs or N2ΔPg; 36 h later, the cells were harvested, homogenized, and centrifuged to remove membrane-associated proteins, and soluble β-catenin was analyzed by immunoblot using an anti-β-catenin antibody. Two different exposures of the blot (5 s and 1 min) are shown. Very low levels of soluble β-catenin were seen in cultures transfected with the pCDNA3 plasmid (empty vector) alone. (F) A comparison of the stabilization of soluble β-catenin by Wnt-1 and cnxPg. 293T cells were cotransfected with plasmids that drove the expression of Wnt-1 (4 μg), S37→F–β-catenin (2 μg), cnxPg-GFP (2 μg), or cnxN4ΔPg-GFP (2 μg) and assayed 36 h later for soluble β-catenin, as in D (exposures of 1 and 5 min are shown). (G) The ability of cnxPg, Wnt-1, and S37→F–β-catenin plasmids to activate OT. 293T cells were transfected with cnxPgs or Wnt-1–expressing plasmids, and their ability to activate OT was measured. In a large number of experiments (>10), Wnt-1 produced only a modest increase in reporter activity, ranging from 2- to 10-fold.

Embryo Experiments

To assay the biological activity of various cnxPg constructs, RNA was synthesized from the T7 promoter of the pT7cnx-GFP plasmids as described previously (Merriam *et al.*, 1997). Fertilized eggs were injected with 10–20 nl of RNA (0.01–0.8 ng/nl). Injected embryos were examined using a Leitz (Midland, Ontario, Canada) M5 stereo dissecting microscope equipped with epifluorescence optics to identify those expressing the exogenous protein. The effects of the injected RNAs on axis formation were assayed at stages 15–20; axis duplication was based on the presence of two distinct neural axes (see Merriam *et al.*, 1997).

Cell Culture, Transfection, and Plasmid Injection

Xenopus A6 cells were cultured on glass coverslips in 85% Leibowitz L15 medium supplemented with 10% FCS and antibiotics and grown at room temperature. Injection of plasmid DNAs (1–10 μg/ml) was carried out as described previously (Klymkowsky, 1999). Green fluorescence was first visible within 2–4 h of injection. Living cells were examined using a Zeiss (Thornwood, NY) IM35 microscope equipped with appropriate filter sets; images were captured on slide film (Ektachrome 400, Kodak, Rochester, NY) or on an Apple (Cupertino, CA) Power Macintosh 6500/275 computer using a Microimage (Boyertown, PA) i308 video camera and the Minimonitor 1.2 program.

Immunofluorescence Microscopy

Cells were fixed with 70% acetone/30% ethanol for 5 min, rehydrated in Tris-buffered saline (TBS), and stained with appropriate antibodies. GFP autofluorescence were visualized directly using fluorescein optics. The mouse anti-plakoglobin (γ-catenin) (Transduction Laboratories, Lexington, KY) mAb was used to visualize untagged plakoglobin-containing polypeptides; its epitope is located in the C-terminal region of plakoglobin and is present in all versions of plakoglobin used here. A rabbit anti-*Xenopus* plakoglobin antibody, obtained from Thomas Kurth and Peter Hausen (Max Planck Institute, Tubingen, Germany), was used in some studies. myc-tagged polypeptides were visualized using the mouse anti-myc mAb 9E10 (Evan *et al.*, 1985). APC was visualized using a rabbit antiserum directed against the C-terminal region of APC (Santa Cruz Biotech, Santa Cruz, CA). Western immunoblot studies, carried out by Richard Nelson and Barry Gumbiner (Sloan-Kettering Memorial Cancer Center, New York, NY) with baculovirus-synthesized *Xenopus* APC, indicate that this anti-APC antiserum reacts specifically with *Xenopus* APC (R. Nelson and B. Gumbiner, personal communication; and our unpublished results). A mouse anti-E-cadherin antibody (Transduction Laboratories) was used to visualize E-cadherin. β-Catenin was visualized using a rabbit anti-β-catenin antibody (supplied by Pierre McCrea, University of Texas, Houston, TX; see McCrea *et al.*, 1993; Fagotto and Gumbiner, 1994). Bound antibodies were recognized using either fluorescein-, Texas Red- (Southwestern Biotechnology, Birmingham, AL), Alexa 488-, or Alexa 495- (Molecular Probes, Eugene, OR) conjugated species-specific secondary antibody conjugates. Cells were mounted in air-vol/glycerol and viewed using a Zeiss IM35 microscope and photographed onto Kodak Ektachrome 400 film, as described by Carl and Klymkowsky (1999). Slides were digitized using a Polaroid (Cambridge, MA) SprintScan 35plus slide scanner. Alternatively, cells were visualized using a Nikon (Tokyo, Japan) upright epifluorescence microscope with a ×100 lens, and the images were captured using a Cooke (Auburn Hills, MI) Sensicam video camera on a PowerMacintosh 8500/150MHz computer using the SlideBook 2.1 program (Intelligent Imaging Innovations, Denver, CO). Images were deconvoluted using the SlideBook program and exported as TIFF files. Images were manipulated using Photoshop 4.0 (Adobe, Mountain View, CA) software, and final figures were prepared using Illustrator 7.0 (Adobe).

Reporter Studies

To measure the ability of various forms of cnxPg to activate a TCF-dependent reporter construct, human 293T cells were cotransfected with the plasmid to be tested, an optimized version of the TOPFLASH reporter plasmid (OT) (Korinek *et al.*, 1997) (supplied by Bert Vogelstein, Johns Hopkins University, Baltimore, MD), and the pCDNA3.1-lacZ plasmid. Transfections were performed in duplicate. For each experiment, 60-mm plates were seeded with equal numbers of cells on the night before transfection. A $CaPO_4$-based transfection method was used for all transfections with Stratagene Stable Mammalian Transfection Kits. Cells were 30–50% confluent at the time of transfection. Cells were harvested 36 h after transfection by lysing in 1× reporter lysis buffer (Promega, Madison, WI). Lysates were pelleted and 10 μl of each supernatant was added to 100 μl of luciferase assay substrate (Promega). Luciferase activity was measured (with duplicate readings for each sample) using a Berthold (Bad Wildbad, Germany) Lumat LB9507 luminometer. Readings were normalized for transfection efficiency by measuring β-galactosidase activity. To monitor the accumulation of cnxPgs, cell lysates were analyzed by immunoblot (see below) using mouse mAbs directed against either plakoglobin (Transduction Laboratories) or GFP (Boehringer-Mannheim, Indianapolis, IN).

Reverse Transcriptase-PCR Analysis of TCFs in 293T Cells

Total RNA was extracted from human 293T cells and Jurkat lymphoma cells using a Qiagen (Valencia, CA) RNeasy kit. The RNA was reverse transcribed into cDNA using a Life Technologies/BRL (Bethesda, MD) one-step reverse transcriptase-PCR kit using 1 μl of RNA at 1 μg/ml, according to the manufacturer's protocol. PCR was carried out under the following cycling conditions: 1 time at 50°C for 3 min, 30 times at 94°C for 2 min, 94°C for 25 s, 56°C for 30 s, and 72°C for 2 min, and 1 time at 72°C for 15 min. PCR products were resolved on a 2% NuSieve FMC (Rockland, ME) agarose gel. The oligonucleotides used for the PCR analysis were: TCF1 5′, 5′ TCA AGA AGC CCC TCA ATG CC 3′; TCF1 3′, 5′ TTG GTG CTT TTC CCT CGA CC 3′; LEF1 5′, 5′ CAG AAG GAA AAG ATC TTC GC 3′; LEF1 3′, 5′ GTA GGA GGG TCC CTT GTT GTA 3′; TCF3 5′, 5′ CAG TCA CAG CAG CAA GTT TAG GAG 3′; TCF3 3′, 5′ GGG TTT CTG GTT TGG TGG TGA AG 3′; TCF4 5′, 5′ TCC AGA GAA GAG CAA GCG AAA TAC 3′; and TCF4 3′, 5′ TGA GGT CTG TAA TCG GAG GAA GTG 3 ′.

Examination of Cytoplasmic β-Catenin Levels

To examine the effects of cnxPgs on cytosolic β-catenin levels, samples were prepared as described by Shimizu *et al.* (1997). Cultures of 293T cells were transfected with 4 μg of plasmid DNA. Thirty-six hours after transfection, cells were harvested in 10 mM Tris (pH 7.5), 140 mM NaCl, and 2 mM DTT supplemented with protease inhibitors. The samples were then scraped from the plates, lysed in a Dounce homogenizer, and centrifuged for 1 h at 100,000 × *g* at 4°C. Supernatants were removed and diluted in SDS-sample buffer. The diluted sample was then separated on a 10% SDS-PAGE gel and transferred to nitrocellulose. After confirming even transfer by staining with Ponceau S, the blot was incubated for 30 min at room temperature in blocking solution (0.1% Tween-20 in TBS with 5% BSA). Blots were incubated for 12–16 h at 4°C with primary antibody against anti-β-catenin (Transduction Laboratories) diluted 1:1,000 in blocking solution. Blots were washed three times (5–10 min per wash) in 0.1% Tween-20 in TBS, incubated in secondary antibody (goat anti-mouse immunoglobulin G–peroxidase), diluted 1:15,000 in blocking solution for 30 min at room temperature, washed three times for 10–15 min in Tween-20 in TBS, exposed to ECL substrate (Amersham, Arlington Heights, IL), and exposed to film.

RESULTS

Three assays are commonly used to study the Wnt signaling activity of a polypeptide: its ability to induce an ectopic dorsal axis in *Xenopus*, to activate a TCF-responsive reporter plasmid, and to stabilize soluble β-catenin. Previously, we found that injection of RNAs encoding membrane-anchored forms of plakoglobin led to the synthesis of cytoplasmic proteins that induced an ectopic dorsal axis in *Xenopus* embryos but had no apparent effect on nuclear β-catenin levels (Merriam *et al.*, 1997). To study further the mechanism of cnxPg action, we created a panel of GFP-tagged (and untagged) cnxPg deletion mutants (Figure 1). The mutations removed the entire N-terminal domain of plakoglobin together with the first (cnxN2ΔPg, starts at Asn-167 of plakoglobin), second (cnxN3ΔPg, starts at His-210), third (cnxN4ΔPg, starts at Leu-250), or fourth (cnxN5ΔPg, starts at Gln-292) Arm repeats (Figure 1). Based on the original definition of the Arm repeats, these deletions leave the last five to eight amino acids of the N-terminal deleted Arm repeat, as defined structurally in β-catenin (Huber *et al.*, 1997).

To compare the signaling activity of cnxPg with that of plakoglobin and β-catenin, we used full-length and deleted versions of plakoglobin (Karnovsky and Klymkowsky, 1995; Rubenstein *et al.*, 1997) and a mutated version (S28→A) of plakoglobin analogous to the S37→A oncogenic mutation described for β-catenin. In addition, the pt-mutant version of *Xenopus* β-catenin (Yost *et al.*, 1996) was also tested. Oncogenic mutations in β-catenin lead to the stabilization of the cytoplasmic form of the protein and greatly enhance its ability in Wnt-signaling assays (see Yost *et al.*, 1996; Morin *et al.*, 1997).

Wnt-like Signaling Activity of cnxPg Requires the N-Terminal Arm Repeat Region

Previously, we found that a unanchored N5Δ version of plakoglobin induced axis duplication in *Xenopus* (Rubenstein *et al.*, 1997). To determine whether the same N-terminal region was required for the signaling activities of anchored plakoglobin, we tested the ability of RNAs encoding the various cnxPg deletion mutations to induce the formation of an ectopic axis when injected into fertilized *Xenopus* eggs. As previously reported, injection of cnxPg-GFP mRNA induced axis duplication (Merriam *et al.*, 1997) (Figure 2A). Injection of cnxN3ΔPg-GFP RNA also induced axis duplication, whereas injection of cnxN4ΔPg-GFP RNA did not (Figure 2A). Based on the intensity of green fluorescence, the products of all tested constructs appeared to accumulate to roughly equal extents. To determine if the N3Δ deletion decreased the efficiency of axis induction, embryos were injected with various amounts of cnxPg-GFP and cnxN3ΔPg-GFP RNAs; cnxN3ΔPg-GFP RNA was less efficient than equivalent amounts of cnxPg-GFP RNA at inducing axis duplication (Figure 2A).

cnxPg Activates a TCF-responsive Reporter and Stabilizes Soluble β-Catenin

We next examined the ability of the cnxPg deletion constructs to transactivate a TCF-responsive reporter, OT, when cotransfected into 293T cells. The OT plasmid contains three copies of a TCF-binding motif (CCTTTGATC) upstream of a

Table 1. Variation in β-catenin/cnxPg activation of OT

Experiment	Normalized OT activation[a]	
	cnxPg	S37 → F–β-catenin
1 1.0 μg of DNA	100.6	120.8
2 1.0 μg of DNA	79.4	89.8
3 1.0 μg of DNA	72.7	122.4
4 0.2 μg of DNA	53.6	100.2
0.5 μg of DNA	73.9	98.9
1.0 μg of DNA	66.3	115.2
2.0 μg of DNA	130.0	99.7

[a] Cells transfected with a Wnt-1–expressing plasmid (1–2 μg of DNA) consistently activated OT between 2- and 10-fold under these conditions.

minimal *c-fos* promoter. All transfections also included a plasmid encoding LacZ (pCDNA3.1-βgal) to normalize for transfection efficiency by measurement of β-galactosidase activity. Reverse transcriptase-PCR analysis of 293T cells indicated that they expressed detectable levels of all four TCF RNAs, i.e., TCF1, LEF1, TCF3, and TCF4; in contrast, human Jurkat cells expressed detectable levels of TCF1 and LEF1 only (Figure 2B). The OT reporter was efficiently activated by a S37→F "stabilized" (oncogenic) mutant form of β-catenin (Figure 2C). cnxPg and cnxN2ΔPg were equally effective, and typically as or more effective than oncogenic β-catenin, at activating the OT reporter (Figure 2C and Table 1). cnxN3ΔPg was significantly less effective, although it still consistently activated OT 5- to 10-fold above background. cnxN4ΔPg and cnxN5ΔPg did not activate the OT reporter. Interestingly, the N2Δ form of plakoglobin (Figure 2C) activated the reporter only weakly, even though it induced axis duplication in *Xenopus* (Rubenstein *et al.*, 1997). A variant of OT containing point mutations that prevent TCF binding to the three TCF-binding motifs was not activated above basal levels by any of the constructs described here (our unpublished results).

Western blot analysis of extracts from cells transfected with these constructs indicated that similar amounts of each polypeptide were produced (Figure 2D). We also assayed the effects of transfection of the various cnxPgs into 293T cells on the levels of soluble β-catenin (Figure 2E). cnxPg and cnxN2ΔPg were equally effective at increasing the concentration of soluble β-catenin, whereas cnxN3ΔPg was less effective and cnxN4ΔPg stabilized soluble β-catenin only very weakly. As reported previously by Miller and Moon (1997) and Simcha *et al.* (1998), expression of wild-type plakoglobin increased soluble β-catenin (our unpublished results); in contrast, expression of N2ΔPg failed to induce a detectable increase in soluble β-catenin (Figure 2E). The ability of cnxPg to stabilize cytosolic β-catenin was similar to that seen in cells transfected with plasmid encoding Wnt-1 (Figure 2F); however, the ability of cnxPg to activate the OT reporter was much greater than that seen with Wnt-1 (Figure 2G). Transfection with the Wnt-1 plasmid increased reporter activity 2- to 10-fold, compared with the 50- to 150-fold activation seen with cnxPg. Therefore, it seems likely that the stabilization of β-catenin is not the only factor that determines the level of signaling induced by cnxPg.

Different TCFs Differ in Their Ability to Activate OT Alone and in Combination with β-Catenin

The response of the OT reporter to β-catenin is presumably mediated by TCFs. TCFs are reported not to activate TCF-dependent reporters on their own, but coexpression of TCFs and β-catenin enhances reporter activation over the levels seen with either alone (see Korinek *et al.*, 1997; Hsu *et al.*, 1998, and references therein). Therefore, if activation of OT by cnxPg were due solely to cnxPg's effects on β-catenin levels, increasing TCF levels should enhance OT activation by cnxPg. To test this hypothesis, we examined the effects of expressing various TCFs on the ability of β-catenin and cnxPg to activate the OT reporter. These studies led to several surprising observations. First, it is clear that different TCFs behaved quite differently in this assay system (Figure 3). Transfection of 293T cells with mLEF1 alone consistently activated OT activity 10- to 20-fold above baseline (Figure 3, A and C), whereas transfection of either hTCF4 (Figure 3E) or XTCF3 (Figure 3G) alone failed to activate OT. Cotransfection of LEF1 or hTCF4 with oncogenic forms of β-catenin resulted in a large increase in OT reporter activation (Figure 3, A and E) well beyond the strong activation induced by oncogenic β-catenin alone. A similar synergistic activation of OT was observed when cells were transfected with the S28→A mutant form of plakoglobin and LEF1 (Figure 3B).

Previously, Molenaar *et al.* (1996) reported that XTCF3 enhanced the ability of β-catenin to activate a TCF-dependent reporter. In contrast, we found that cotransfection of XTCF3 and either the human or *Xenopus* β-catenin suppressed β-catenin's ability to activate OT by approximately twofold (Figure 3G). DNA-binding studies indicate that XTCF3 binds to β-catenin and that the XTCF3/β-catenin complex binds to a TCF-DNA consensus sequence (our unpublished results). The reason behind the discrepancy between our results and those of Molenaar *et al.* (1996) is unclear, but the ability of XTCF3 to suppress β-catenin signaling is consistent with its activity in the *Xenopus* embryo (see below).

cnxPg Does Not Activate OT Simply by Stabilizing β-Catenin

Contrary to what would have been expected if cnxPg activated OT solely by increasing the level of cytosolic β-catenin, coexpression of cnxPg with either mLEF1 (Figure 3C) or hTCF4 (Figure 3F) suppressed cnxPg's ability to activate OT. XTCF3's effect on cnxPg was similar to its effect on β-catenin, i.e., coexpression of XTCF3 suppressed activation of the OT reporter by cnxPg (Figure 3H). The inhibitory effects of TCFs on cnxPg reporter activity were not due to effects on cytosolic levels of β-catenin, because cotransfection with mLEF1 or XTCF3 did not affect the levels of cytosolic β-catenin induced by cnxPg (Figure 3D).

cnxPg Alters the Intracellular Distribution of β-Catenin, Axin, and APC

To gain more insight into the actions of cnxPgs on signaling, we tested their abilities to alter the localization of various

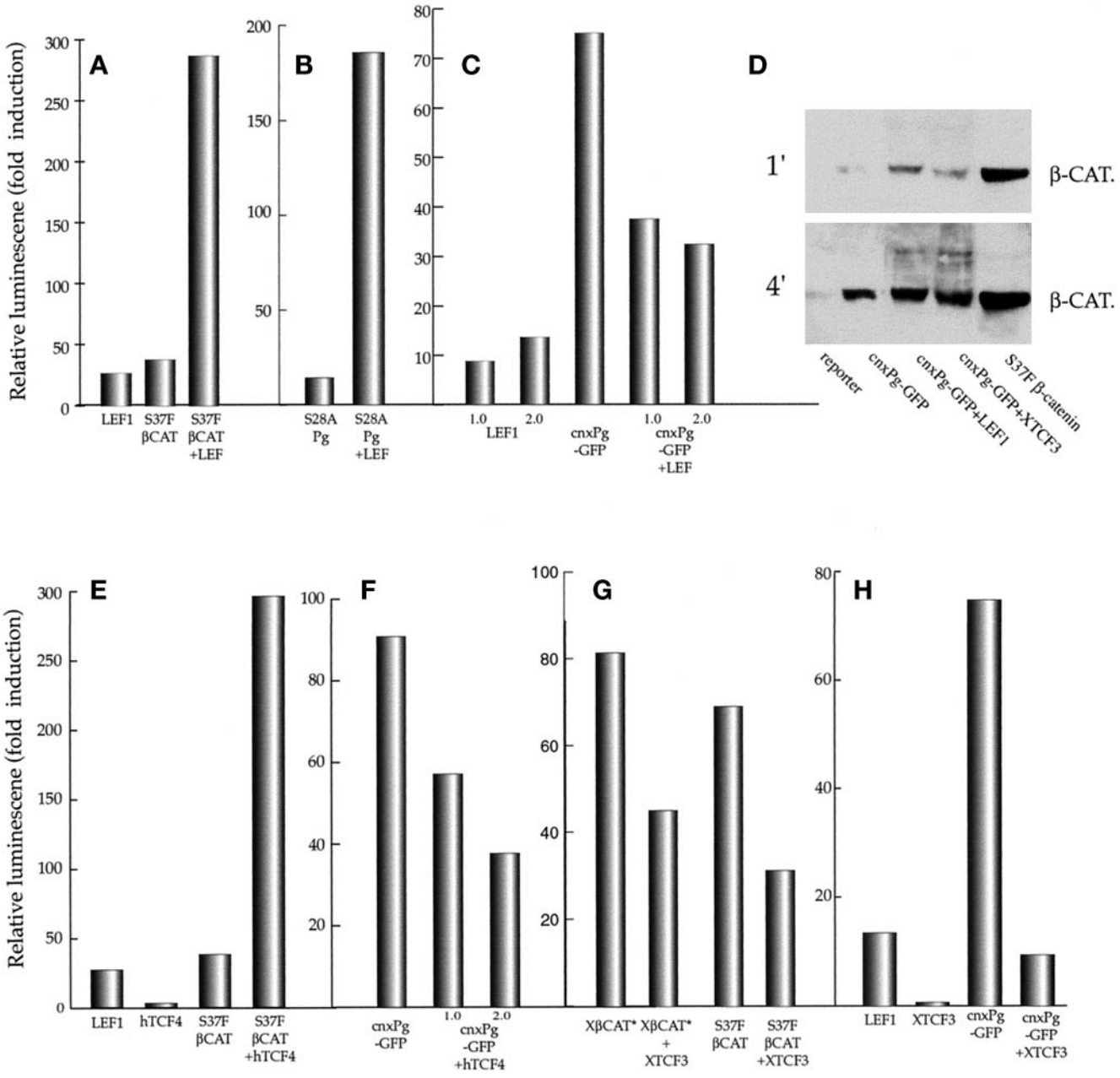

Figure 3. (A–C) Interactions between β-catenin, cnxPg, and TCFs. (A) 293T cells were transfected with plasmids driving the expression of either mouse LEF1 (2 μg), S37→F–β-catenin (2 μg), or both plasmids together, and their ability to activate the OT reporter was measured. A strong synergy between LEF1 and S37→F–β-catenin was seen. (B) A similar synergy in the activation of the OT reporter was observed when S28→A plakoglobin (S28A Pg) (1 μg) was coexpressed with mouse LEF1 (1 μg). (C) In contrast, cotransfection of 293T cells with mouse LEF1 plasmid DNA (1 or 2 μg) suppressed the activation of OT by cnxPg-GFP plasmid (1 μg). This pattern of reporter activity was seen reproducibly in at least three independent experiments. (D) Effect of TCF and cnxPg coexpression on soluble β-catenin levels. 293T cells were transfected with S37→F–β-catenin, cnxPg-GFP plasmid DNA, or both cnxPg-GFP and LEF1 or XTCF3 plasmid DNAs. After 36 h, the cells were homogenized and soluble β-catenin was assayed by immunoblot. Two exposures (1 and 4 min) of the blot are shown. (E–H) Differences in the activities of TCFs. (E) On its own, hTCF4 (1 μg) did not activate the OT reporter but did produce a strong enhancement of the activity of S37→F–β-catenin (2 μg). (F) Coexpression of hTCF4 and cnxPg-GFP suppressed cnxPg's ability to activate the OT reporter. (G) XTCF3 (2 μg) suppressed the ability of both the pt-mutant form of *Xenopus* β-catenin (2 μg) (described by Yost *et al.*, 1996) and the oncogenic mutant form of human β-catenin (2 μg) to activate the reporter. (H) XTCF3 (1 μg) did activate OT on its own, and coexpression of XTCF3 (1 μg) suppressed cnxPg-GFP's ability to activate OT. In E and H, the effects of transfecting mLEF1 (1 μg) are included for the purpose of comparing the various experiments. The patterns of reporter activity illustrated here were seen reproducibly in at least three independent experiments.

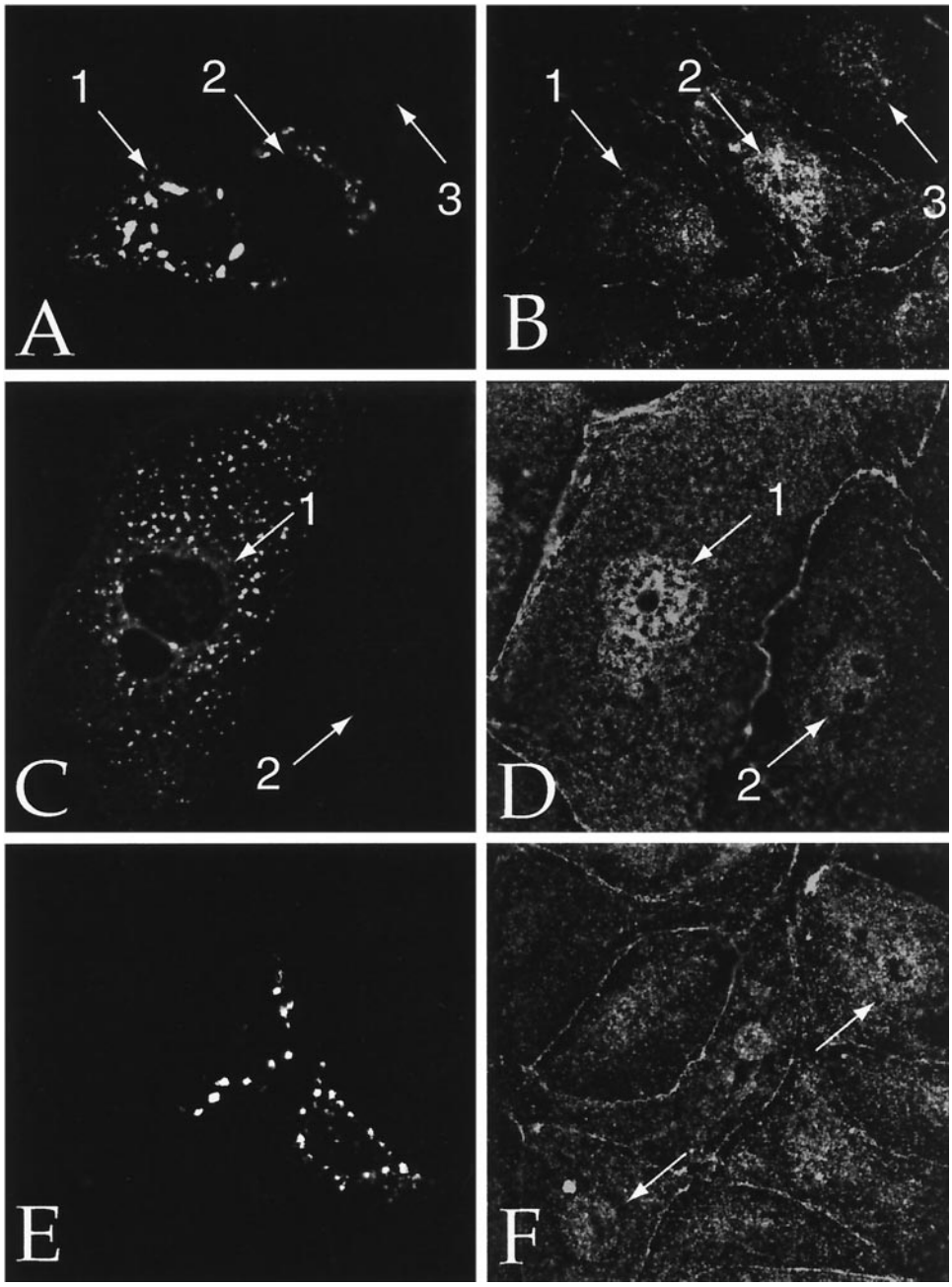

Figure 4. Localization of β-catenin in cells expressing cnxPgs. *Xenopus* A6 cells were injected intranuclearly with DNAs encoding cnxPg (A and B), cnxN3ΔPg (C and D), or cnxN4ΔPg (E and F); 20 h later, the cells were fixed and stained for plakoglobin with a mouse anti-plakoglobin mAb and an Alexa 596–conjugated secondary antibody (A, C, and E) and for β-catenin with a rabbit anti-β-catenin antiserum and an Alexa 488–conjugated secondary antibody (B, D, and F). A clear increase in nuclear β-catenin was seen in many cells expressing cnxPg (arrow 2 in B); however, there was variability in the effects observed. For example, a cell expressing high levels of cnxPg (arrow 1) could show little apparent increase in nuclear β-catenin compared with a neighboring cell expressing lower levels (arrow 2) or compared with an uninjected cell (arrow 3). In cells expressing cnxN3ΔPg (C), there was often an apparent increase in nuclear β-catenin (D, arrow 1; an uninjected cell is marked by arrow 2); no such increase in nuclear β-catenin (F) was seen in cells expressing cnxN4ΔPg (E) (arrows in F indicate the level of nuclear β-catenin in uninjected cells). All images have been deconvoluted.

Wnt signaling components in *Xenopus* A6 cells. Because transfection efficiencies are low in *Xenopus* cells by conventional methods, we injected DNAs encoding cnxPgs directly into nuclei (see Klymkowsky, 1999). The encoded cnxPgs tended to form irregular cytoplasmic aggregates and did not appear to enter the plasma membrane efficiently (Figures 4A and 5, G and J). Occasionally, cnxPgs were seen in a reticular pattern (see Figure 8, E and I, for examples) likely to represent the endoplasmic reticulum (our unpublished results). In contrast, unanchored forms of plakoglobin accumulate largely in the nuclei in living and fixed cells (Karnovsky and Klymkowsky, 1995; Merriam *et al.*, 1997; Rubenstein *et al.*, 1997) (Figure 5D).

Consistent with our biochemical studies of soluble β-catenin (see above) and the observations of Miller and Moon (1997; see also Hsu *et al.*, 1998), A6 cells injected with cnxPg DNA often displayed increased levels of nuclear β-catenin (Figure 4). However, this effect was quite variable between cells and did not always correlate with the level of cnxPg expression (Figure 4, A–C). It is perhaps this variability that obscured an increase in nuclear β-catenin in embryonic cells expressing cnxPg (see

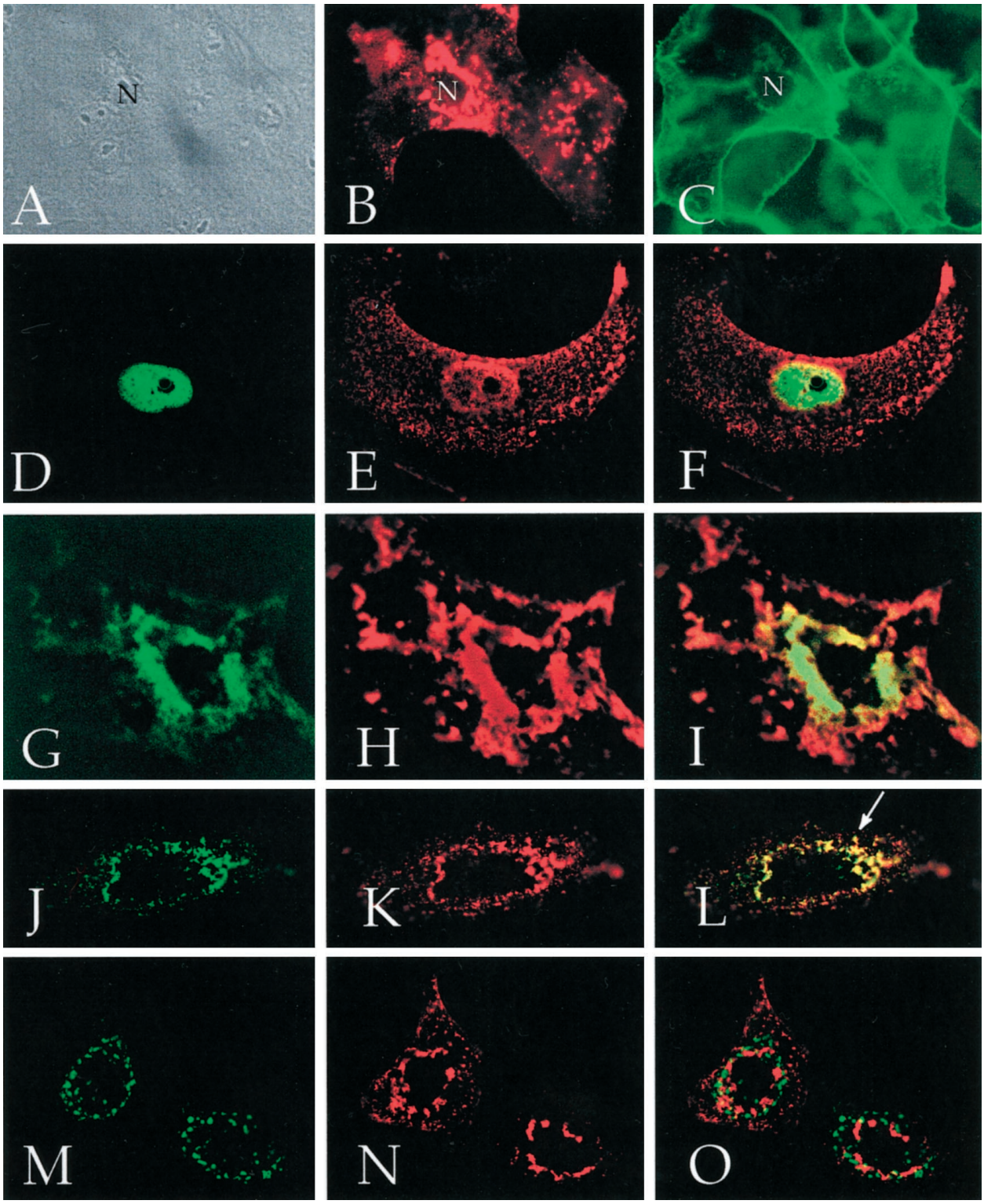

Figure 5. cnxPg colocalizes with exogenous Axin. To examine the colocalization between cnxPgs and Axin, *Xenopus* A6 cells were injected with plasmid DNAs encoding mtAxin either alone (A–C) or together with N2Δ-plakoglobin (D–F), cnxPg-GFP (G–I), cnxN3ΔPg-GFP (J–L), or cnxN4ΔPg-GFP (M–O); 20 h later, the cells were fixed and stained with the anti-myc antibody 9E10. In cells injected with mtAxin DNA alone, the protein accumulated in irregular cytoplasmic aggregates (A, phase image; B, staining with anti-myc antibody; C, staining with a rabbit anti-plakoglobin antibody to reveal cell boundaries). In cells coinjected with N2Δ-plakoglobin-GFP and mtAxin-encoding plasmids, the

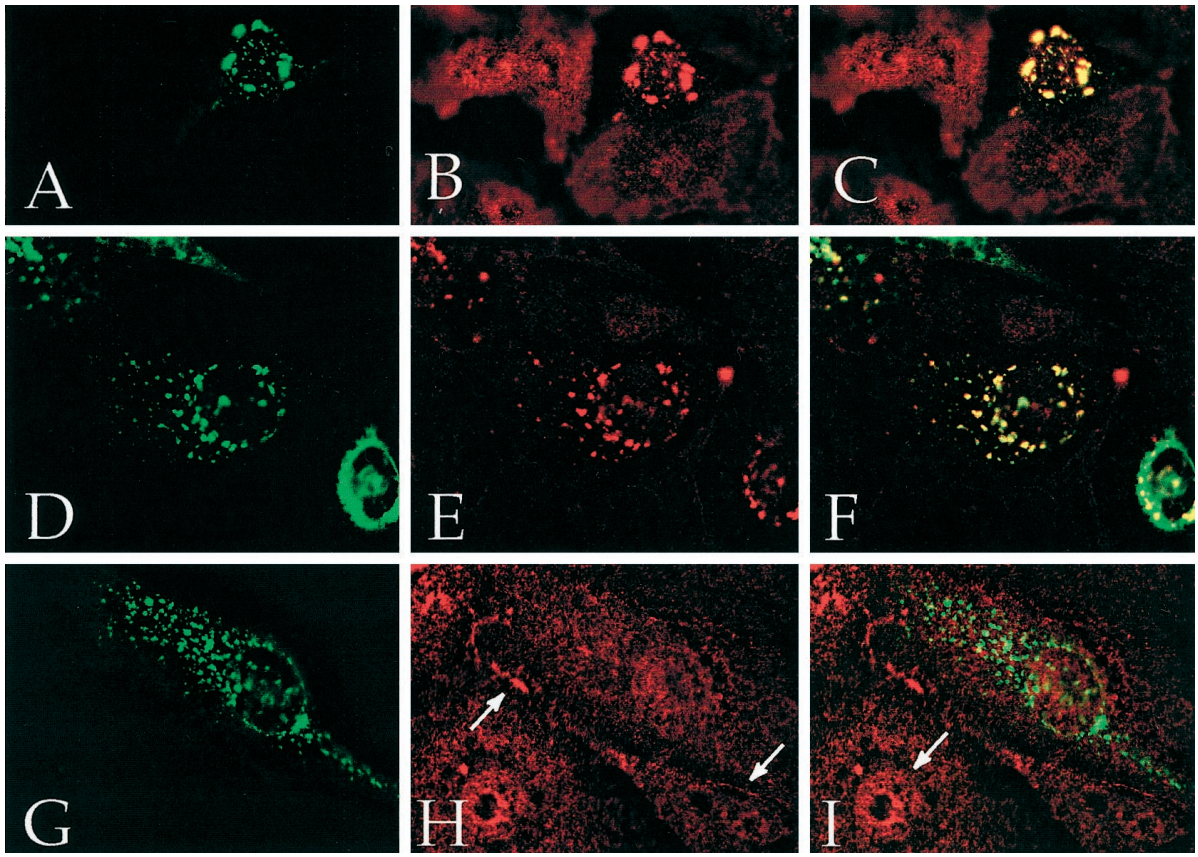

Figure 6. cnxPg colocalization with APC. The nuclei of *Xenopus* A6 cells were injected with DNAs encoding various cnxPgs. After ~16–18 h, the cells were fixed and stained for APC using an Alexa 596–conjugated secondary antibody. cnxPg-GFP (A), visualized through its green fluorescence, was found to induce the reorganization of endogenous APC (B) (C shows an overlap of A and B). Endogenous APC was found to colocalize with cnxN3ΔPg-GFP (D, green fluorescence; E, APC staining; F, overlap) but not with cnxN4ΔPg-GFP (G, green fluorescence; H, APC staining; I, overlap). Staining for APC was also found within nuclei of some cells (I, arrow). Punctate anti-APC staining at cell boundaries can be seen in some images (H, arrows). All images were deconvoluted, and areas of yellow in C, F, and I correspond to regions of overlap.

Merriam *et al.*, 1997). Injection of the cnxN3ΔPg plasmid increased nuclear β-catenin in some cells (Figure 4, C and D), whereas expression of the cnxN4ΔPg-GFP polypeptide had no apparent effect on β-catenin's intracellular localization (Figure 4, E and F).

Axin and the related protein Conductin/Axil bind to β-catenin in a complex with APC and Gsk3β (see above). To determine whether Axin could colocalize with cnxPgs, we coinjected DNAs encoding wild-type mtAxin (Zeng *et al.*, 1997) into A6 cells either alone (Figure 5, A–C) or together with DNA encoding either N2ΔPg-GFP (Figure 5, D–F) or cnxPgs (Figure 5, G–O). After injection of mtAxin DNA alone, mtAxin was found in irregular aggregates scattered throughout the cytoplasm and excluded from the nucleus (Figure 5, A–C). When DNAs encoding mtAxin and N2ΔPg-GFP were coinjected, green fluorescence from the N2ΔPg-GFP polypeptide was almost entirely nuclear, whereas mtAxin was found in aggregates scattered throughout the cytoplasm and within the nuclei (Figure 5, D–F). When mtAxin and cnxPg-GFP DNAs were coinjected, the two polypeptides colocalized (Figure 5, G–I). Deletion of plakoglobin's N-terminal head and first two Arm repeats (cnxN3ΔPg-GFP) did not disrupt the colocalization of mtAxin with cnxPg (Figure 5, J–L), whereas deletion of the third Arm repeat (cnxN4ΔPg-GFP) abolished colocalization (Figure 5, M–O). In human 293T cells, coexpression of mtAxin with cnxPg suppressed cnxPg's ability to activate the OT reporter by 40–60% (our unpublished results).

Figure 5 (cont.) N2Δ-plakoglobin–associated green fluorescence (D) was localized to nuclei; mtAxin (E) was partially localized to nuclei and to the nuclear periphery (F shows an overlap of D and E). In contrast, when mtAxin was coinjected with cnxPg-GFP (G–I), cnxN2ΔPg-GFP (our unpublished results), or cnxN3ΔPg-GFP (J–L) plasmid DNAs, there was extensive colocalization of cnxPg-associated green fluorescence (G and J) and mtAxin (H and K) (I shows an overlap of G and H, and L shows an overlap of J and K). In contrast, when mtAxin and cnxN4ΔPg DNAs were coinjected (M–O), no overlap was observed between cnxN4ΔPg-GFP–associated green fluorescence (M) and the pattern of anti-myc staining (N) (O shows an overlap of M and N). All images except A–C were deconvoluted, and areas of yellow in F, I, L, and O correspond to regions of overlapping fluorescence.

APC forms a complex with β-catenin or plakoglobin (see above), targeting these catenins for proteolytic degradation. In *Xenopus* A6 cells, anti-APC antibody stained both nuclei and cytoplasm diffusely, and staining of the cell boundary was sometimes visible (Figure 6, H and I) (see Neufeld and White, 1997; Senda *et al.*, 1998). In cells expressing cnxPg-GFP (Figure 6, A–C), anti-APC staining colocalized with cnxPg-GFP. APC colocalized with cnxN3ΔPg (Figure 6, D–F) but not with cnxN4ΔPg (Figure 6, G–I) or cnxN5ΔPg (our unpublished results).

cnxPg Does Not Induce the Redistribution of Either E-Cadherin or Dishevelled

Chitaev *et al.* (1996) showed that membrane-anchored forms of plakoglobin were capable of interacting with desmosomal cadherins, whereas Cox *et al.* (1999) reported that anchored forms of Armadillo localized to the plasma membrane and coimmunoprecipitated with DE-cadherin. However, cnxPgs do not efficiently reach the plasma membrane in A6 cells (see above), and double-label immunofluorescence microscopy revealed little overlap between cnxPgs and E-cadherin (Figure 7, A and B).

Genetic studies indicate that Dishevelled is activated in response to Wnt signaling and plays a role in the inhibition of Gsk3β. To determine if the expression of cnxPg altered the distribution of Dishevelled, we coexpressed cnxPg-GFP with a myc-tagged form of *Xenopus* Dishevelled (Sokol, 1996) in A6 cells. Exogenous Dishevelled was distributed throughout the cytoplasm and excluded from the nucleus (Figure 7C). The presence of cnxPg had no apparent effect on the distribution of Dishevelled (Figure 7, E–G).

cnxPgs Can Sequester TCFs in the Cytoplasm of A6 Cells

We have previously proposed that cnxPgs act, at least partially, by sequestering and inhibiting negatively acting factors in the cytoplasm. The ability of cnxPgs to sequester Axin and APC supports such a model. To determine whether cnxPgs could also sequester TCFs in *Xenopus* cells, we coexpressed them with either a myc-tagged form of XLEF1 or myc- and GFP-tagged forms of XTCF3 and hLEF1 (Figure 1). When expressed on their own, XLEF1 (Figure 7D) and XTCF3 (Figure 8C) were exclusively nuclear. When DNAs encoding XLEF and cnxPg (Figure 7, H–J) or XTCF3 and cnxPg (our unpublished results) were coinjected, a significant amount of fluorescent signal was observed in the cytoplasm. Cytoplasmic localization of exogenous TCFs was observed in cells expressing cnxN2ΔPg (Figure 8, A and B) or cnxN3ΔPg (Figure 8D) but not in cells expressing cnxN4ΔPg (Figure 8, E and F) or cnxN5ΔPg (our unpublished results). This cytoplasmic localization of exogenous TCFs was not an artifact of fixation, because it could be readily observed in living cells (Figure 8D).

The colocalization of XTCF3 and β-catenin requires the presence of the "catenin-binding domain" located at the N terminus of XTCF3 and LEF1 (Molenaar *et al.*, 1996). A version of XTCF3 with this domain deleted (mtΔN_{166}XTCF3-GFP) failed to colocalize with cnxPg (Figure 7, G and H). Similarly, we found no apparent colocalization between cnxPg and tagged forms of the cytoplasmic tail domain of *Xenopus* Notch, the zinc-finger transcription factor XSlug, or the bHLH transcription factor NeuroD (our unpublished results), all of which localized to nuclei. This finding suggests that the colocalization between cnxPg and TCFs is not due to nonspecific interactions between highly expressed proteins in these cells.

DISCUSSION

Recently, our understanding of the mechanisms underlying Wnt signaling has deepened considerably. After the discovery of the interaction between β-catenin or plakoglobin and TCFs, it was commonly held that β-catenin and plakoglobin acted exclusively as transcriptional coactivators. It was already clear, however, that such a model did not accurately describe one important model system of Wnt-like signaling, i.e., dorsal axis formation in *Xenopus* (see Klymkowsky, 1997). XTCF3, the only TCF so far detected in the early embryo at the time of dorsal determination (see Molenaar *et al.*, 1998), does not by itself induce axis duplication. In fact, overexpression of XTCF3 partially suppresses dorsal axis formation (Zorn *et al.*, unpublished observations), and a mutated form of XTCF3, missing its catenin-binding domain, blocks dorsal differentiation (Molenaar *et al.*, 1996). Analysis of the promoter region of the homeobox gene *Siamois*, a target of Wnt/β-catenin transcriptional regulation in the early *Xenopus* embryo (Carnac *et al.*, 1996), indicates that XTCF3 represses expression ventrally but does not appear to stimulate expression dorsally (Brannon *et al.*, 1997; see also Fan *et al.*, 1998). In contrast to Molenaar *et al.* (1996), we found that XTCF3 fails to enhance activation of the TCF-responsive OT reporter by β-catenin, but rather suppresses it (Figure 3G). The origin of this discrepancy is unclear. We have confirmed the sequence of the XTCF3 construct used in our studies and shown that XTCF3 binds to a TCF DNA consensus sequence and that the XTCF3/DNA complex binds to β-catenin (our unpublished results). Perhaps differences in the TCF-responsive reporters and cell lines used are responsible, although the repressive effects of XTCF3 on OT activity are more consistent with the effects of XTCF3 overexpression observed in the *Xenopus* embryo (see Molenaar *et al.*, 1996; Klymkowsky, 1997). In any case, the negative effects of XTCF3 are consistent with a model in which anchored forms of plakoglobin induce Wnt-like axis duplication in *Xenopus*, at least in part, by repressing XTCF3 activity, thereby allowing the ectopic expression of *Siamois* and other directly dorsalizing genes (Figure 9) (see Klymkowsky, 1997).

cnxPg Stabilizes Endogenous β-Catenin

The proposal that membrane-anchored forms of plakoglobin or β-catenin act solely by inhibiting the activity of XTCF3 has been challenged. Miller and Moon (1997) provided clear evidence that expression of a membrane-anchored β-catenin leads to the accumulation of soluble β-catenin, presumably by inhibiting the β-catenin turnover system (see also Hsu *et al.*, 1998; Simcha *et al.*, 1998). Although we previously did not detect an increase in nuclear β-catenin levels in embryonic cells expressing anchored plakoglobins (Merriam *et al.*, 1997), it now seems likely that the methodologies used were not sensitive enough to detect the small changes in β-catenin levels involved (see Figure 4 for example), particularly in the presence of the high basal levels of nuclear β-catenin present at the embryonic stage examined. Our own subsequent stud-

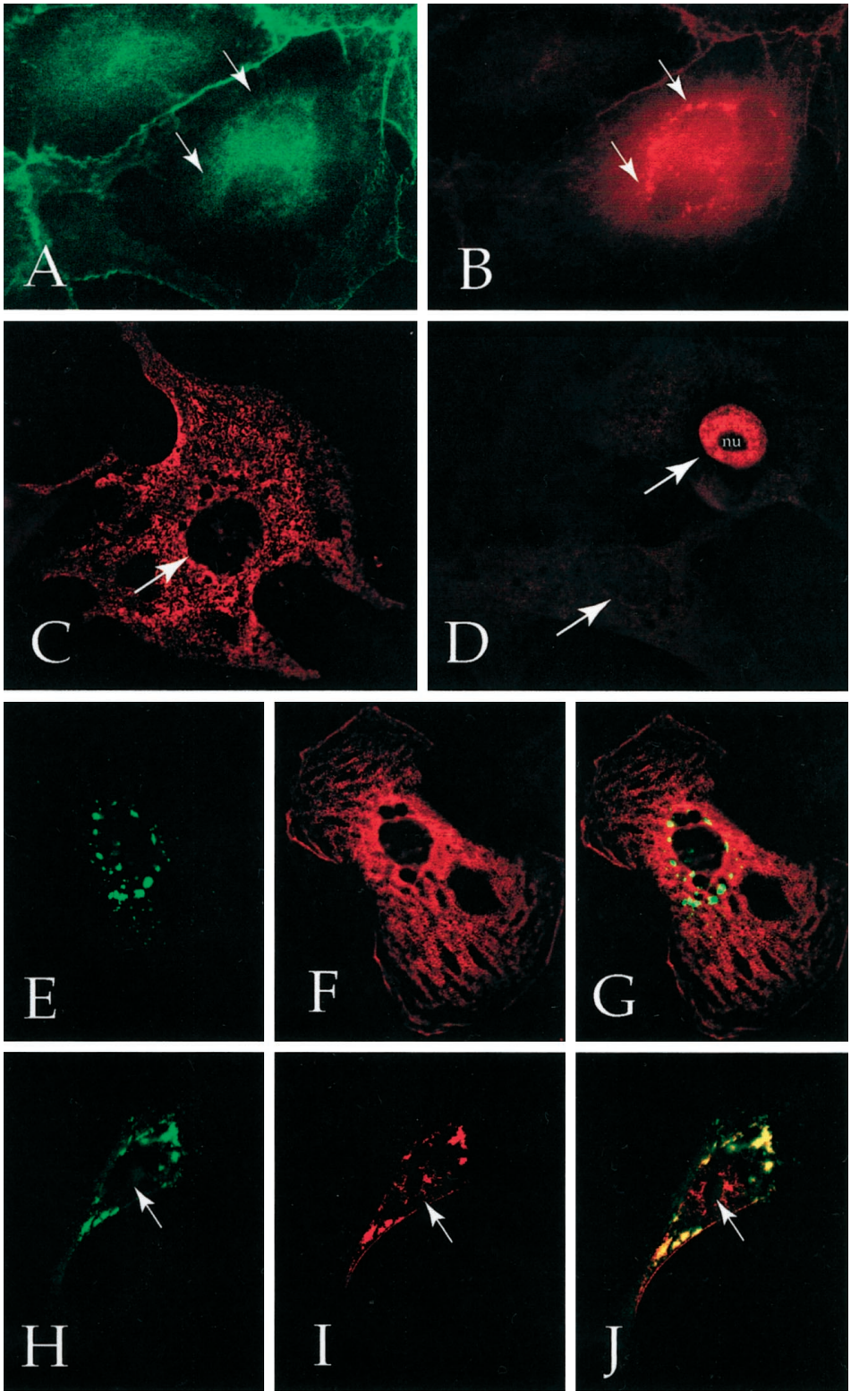

Figure 7. Analysis of colocalization between cnxPg, E-cadherin, Dishevelled, and XLEF-1. *Xenopus* A6 cell nuclei were injected with DNAs encoding cnxPg (A and B), mtXDvl (C), mycXLEF1 (D), cnxPg-GFP and mtXDvl (E–G), or cnxPg-GFP and mycXLef1 (H–J); 20 h later, the cells were fixed and stained. Cells injected with cnxPg DNA were stained with an anti-E-cadherin mAb (A) and a rabbit anti-plakoglobin antibody (B). Cytoplasmic aggregates of cnxPg (B, arrows) did not overlap with intracellular E-cadherin staining (A). mtXDvl (C) was exclusively cytoplasmic, whereas mycXLEF1 was found exclusively in the nucleus (D) (arrows in C and D point to nuclei). When cnxPg-GFP and mtXDvl DNAs were coinjected, the cnxPg-GFP (E, visualized through its autofluorescence) showed no tendency to colocalize with the mtXDvl (F; G shows an overlap of E and F). In contrast, when cnxPg-GFP and mycXLEF1 DNAs were coinjected, the cnxPg-GFP (H, visualized through its autofluorescence) appeared to overlap with the mycXLEF staining (I; J shows an overlap of H and I). In the most extreme examples, such as that illustrated here, the redistribution of XLEF1 was dramatic, such that the nuclear localization of mycXLEF1 was significantly reduced (arrows point to the position of the nucleus). Images in A and B are standard photomicrographs, whereas images in C–J were deconvoluted.

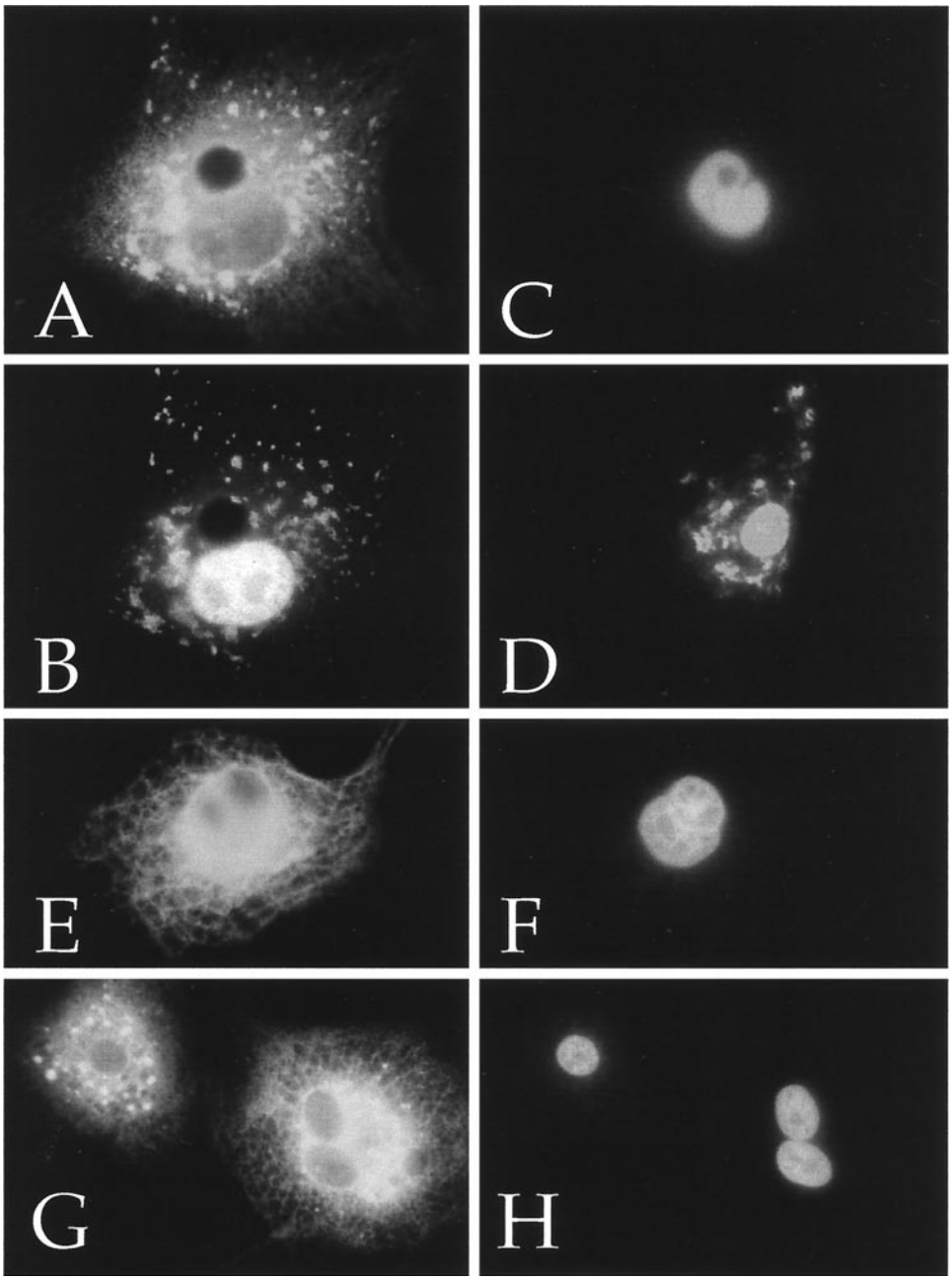

Figure 8. Cytoplasmic localization of XTCF3 associated with the coexpression of cnxPg. Cells were injected with DNA encoding GFP-tagged XTCF3 either alone (C) or together with cnxN2ΔPg (A and B), cnxN3ΔPg (D), or cnxN4ΔPg (E and F); 20 h later, cells were examined while living (C and D) or were fixed and stained with an anti-plakoglobin antibody to visualize the cnxPg polypeptide (A and E). When coexpressed in A6 cells with full-length cnxPg (our unpublished results), cnxN2ΔPg (A and B) or cnxN3ΔPg (D), GFP-XTCF3–derived green fluorescence was observed in the cytoplasm. Cytoplasmic localization of GFP-tagged XTCF3 was not observed in cells expressing XTCF3 alone (C) or in cells coexpressing cnxN4ΔPg (E and F). Removal of the N-terminal 166 amino acids of XTCF3 abolished its interaction with cnxPg (G, stained for plakoglobin; H, green fluorescence). The images in this figure were not deconvoluted.

ies, presented here, confirm that anchored plakoglobins do stabilize cytosolic β-catenin in both *Xenopus* A6 (Figure 4) and human 293T cells (Figure 2D), presumably by interacting with negative factors, i.e., APC (Miller and Moon, 1997) (Figure 6) and Axin (Figure 5). Thus, the idea that anchored plakoglobins or β-catenins act, at least in part, by stabilizing soluble β-catenin is well established.

Our analysis indicates that the N-terminal head and the first Arm repeat of plakoglobin can be removed from cnxPg without abolishing the ability to colocalize with Axin and APC (Figures 5 and 6). Removal of the second Arm repeat (cnxN3ΔPg), however, impaired the ability of the polypeptide to induce axis duplication (Figure 2A), activate the OT reporter (Figure 2C), and stabilize β-catenin (Figure 2E). Removal of the third Arm repeat (cnxN4ΔPg) completely abolished axis duplication activity and the ability to activate the OT reporter and greatly reduced, although it did not eliminate, the stabilizing effect on β-catenin (Figure 2, A, C, and E). The cnxN4ΔPg and cnxN5ΔPg polypeptides also failed to colocalize with either Axin or APC (Figures 5, M–O, and 6, G–I). Interestingly, a N5Δ version of plakoglobin induces axis duplication in *Xenopus* (Rubenstein *et al.*, 1997), arguing for differences in the Wnt-signaling mechanisms of the "free" and anchored forms of the protein.

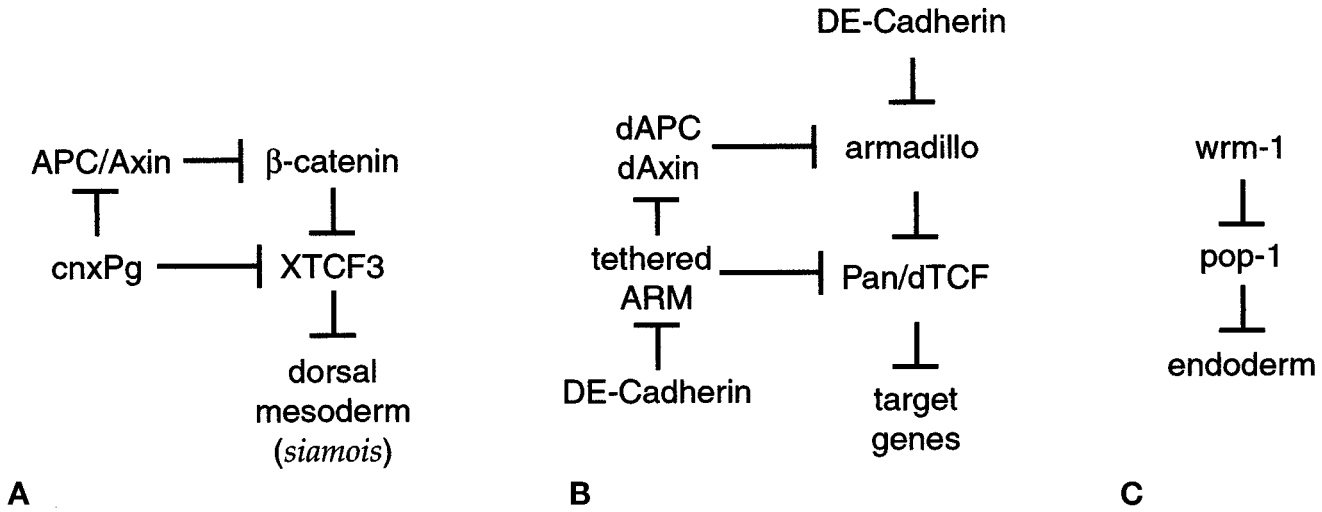

Figure 9. A model of cnxPg action. (A) In the early *Xenopus* embryo, XTCF3 appears to repress the ventral expression of dorsalizing genes (e.g., *Siamois*); on the dorsal side of the embryo, increased levels of β-catenin act to suppress the repressive action of XTCF3. cnxPgs stabilize β-catenin through interactions with components of the β-catenin turnover system, i.e., APC and Axin. They can also act directly on TCFs by sequestering them in the cytoplasm. (B) In *Drosophila*, tethered armadillo inhibits the armadillo turnover system, leading to increased levels of armadillo, interactions with pangolin/dTCF (Pan/dTCF), and altered gene expression. Tethered armadillo has been shown to interact strongly with DE-cadherin (Cox *et al.*, 1999). In the absence of wild-type armadillo, the interaction between tethered armadillo and cadherins would be expected to increase, thereby suppressing the ability of tethered armadillo to interact with Pan/dTCF. In contrast, cnxPg does not appear to interact significantly with E-cadherin in A6 cells (see Figure 7, A and B). The idea that cnxPg could sequester TCFs in the cytoplasm of vertebrate cells is similar to the pathway (C) proposed for Wnt signaling in the early *C. elegans* embryo by Thorpe *et al.* (1997) and Rocheleau *et al.* (1997). Based on the data presented here, we suggest that cnxPg can contribute to the induction of dorsal mesoderm by stabilizing β-catenin and sequestering XTCFs or other negatively acting factors.

cnxPg Does More Than Stabilize β-Catenin

Our analysis of cnxPg in vertebrate cells suggests that cnxPgs do not act exclusively through effects on β-catenin. First, expression of either cnxPg or Wnt-1 in 293T cells results in similar levels of soluble β-catenin (Figure 2F). A comparable stabilization of β-catenin in NIH 3T3 cells by membrane-tethered β-catenin or Wnt-1 was observed by Hsu *et al.* (1998). Despite similar effects on cytosolic β-catenin levels, anchored plakoglobins transactivated the OT reporter to levels 10- to 20-fold greater than did Wnt-1 (Figure 2G and Table 1).

Second, work from several laboratories has shown that β-catenin and LEF1 or TCF4 act synergistically to transactivate OT and other similar reporters when cotransfected into a number of cell types (see Korinek *et al.*, 1997; Hsu *et al.*, 1998). We confirmed these observations by showing that an oncogenic form of β-catenin, when coexpressed with LEF1 or hTCF4, strongly induced reporter activity (at least 4-fold above the levels induced by β-catenin alone and up to 300-fold over baseline activity) (Figure 3, A and E).

Miller and Moon (1997) suggested that plakoglobin acts to induce axis duplication indirectly through effects on β-catenin stability. In cultured cells, expression of plakoglobin stabilizes endogenous β-catenin (Simcha *et al.*, 1998), and coexpression of plakoglobin with LEF1 produces a similar synergistic activation of the OT promoter (Figure 3B). Although this may be true for full-length plakoglobin, it does not seem to be the case for deleted forms of the protein, because the N2Δ form of plakoglobin induces axis duplication (Rubenstein *et al.*, 1997) but does not appear to alter cytoplasmic β-catenin levels (Figure 2E).

If cnxPg's signaling activity were due solely to its ability to increase β-catenin levels, we would predict a similar synergism in OT activation between cnxPg and TCFs. Instead, coexpression of cnxPg and LEF1 or hTCF4 inhibited cnxPg-induced OT transactivation (Figure 3, C and F). One possible mechanism to explain such an inhibition is that the binding of TCFs to cnxPg blocks the interaction between cnxPg, Axin, and APC, leading to a decrease in cytoplasmic β-catenin. This does not appear to be the case, however. Coexpression of LEF1 or XTCF3 and cnxPg does not appear to decrease the cnxPg-induced stabilization of β-catenin (Figure 3D).

Cytoplasmic Sequestration of TCFs by cnxPg

One potential explanation for the behavior of cnxPg emerges from the model we originally proposed, namely, that cnxPg alters gene expression, at least in part, by sequestering in the cytoplasm a molecule that normally represses relevant target genes involved in *Xenopus* axis induction (Figure 9). A growing body of evidence supports the idea that TCFs, and XTCF3 (Figure 2G) in particular, have the characteristics of such a molecule (see above; Klymkowsky, 1997; Bienz, 1998). Mutation of TCF sites in the *Ultrabithorax* and *Siamois* promoters of *Drosophila* and *Xenopus*, respectively (Brannon *et al.*, 1997; Riese *et al.*, 1997; Fan *et al.*, 1998), derepresses expression of these genes in cells not exposed to Wingless/Wnt signals. Furthermore, Groucho-like corepressor proteins bind to XTCF3 and, as a complex, XTCF3 and Groucho can constitutively repress transcription at promoters with TCF-binding sites (Roose *et al.*, 1998).

To examine the possibility that cnxPgs can bind to and sequester TCF-type transcription factors in the cytoplasm, we examined the behavior of myc- and GFP-tagged forms of XLEF1 and XTCF3. Nuclear when expressed on their own (Figures 7D and 8C), both XLEF1 (Figure 7, H–J) and XTCF3 (Figure 8, B and D) were found to accumulate to readily detectable levels in the cytoplasm of cells expressing cnxPg. The cytoplasmic localization of GFP-tagged TCFs could be observed in living cells (Figure 8D), ruling out any artifactual redistribution of the protein during fixation. Staining of fixed cells showed a close association between exogenous TCFs and cnxPgs (Figures 7, H–J, and 8, A and B).

The specificity of the colocalization between cnxPg and TCFs was demonstrated in two ways. The colocalization of TCFs and β-catenin involves the N-terminal region of these DNA-binding proteins (Behrens *et al.*, 1996; Molenaar *et al.*, 1996). When a mutated version of XTCF3 lacking the N-terminal 166 amino acids was tested, it did not colocalize with cnxPg (Figure 8, E and F). We have tested a number of other identically tagged transcription factors and nuclear proteins, including the *Xenopus* zinc-finger transcription factor XSlug, the cytoplasmic domain of the *Xenopus* Notch homologue Xotch, and the mouse HLH transcription factor NeuroD. None of these polypeptides showed any tendency to colocalize with cnxPg (our unpublished results). Based on these data, we conclude that cnxPg specifically interacts with, and can sequester, exogenous TCFs in the cytoplasm of *Xenopus* cells. Because Wnt-1 and cnxPg induce similar increases in β-catenin levels (Figure 2F) but differ significantly in their signaling activity (as measured by OT activation) (Figure 2G), we suggest that inhibition of the repressive activities of TCF family members may, at least in part, account for this difference. Alternatively, cnxPg may either activate some other activator or inhibit some other repressor of TCF signaling, distinct from β-catenin, APC, Axin, or TCF.

How does the ability of cnxPg to sequester a portion of exogenous TCFs in somatic cells relate to its effects in the *Xenopus* embryo? This question clearly requires an analysis of the effects of cnxPg on endogenous TCFs; we are currently generating anti-XTCF3 antibodies to directly examine this issue. However, it is clear that in somatic cells cnxPgs can sequester at least a portion of total exogenous TCFs in the cytoplasm. In the early *Xenopus* embryo, which breaks down its nuclei every 30–40 min during the period of axis determination (Newport and Kirschner, 1982), the likelihood that cnxPgs can significantly affect the intracellular distribution of endogenous XTCF3 seems even more likely.

The Dual Mechanism of cnxPg Action

Based on all of the data presented here, we propose that cnxPg acts by two distinct mechanisms to affect Wnt signaling (Figure 9A). It inhibits the negatively acting cytoplasmic proteins Axin and APC (Figures 5 and 6), thereby stabilizing β-catenin (Figures 2 and 4). This is the mechanism originally proposed by Miller and Moon (1997). On the other hand, cnxPg can also clearly sequester exogenous TCFs to the cytoplasm of *Xenopus* A6 cells (Figures 7 and 8), and its ability to activate the OT reporter is inhibited, rather than activated, by TCF coexpression (Figure 3), suggesting that TCFs may compete for binding sites on cnxPg for negatively acting factors. Clearly, it is the relative levels of specific TCFs expressed in a specific cell type, combined with their relative affinities for β-catenin, plakoglobin, and other accessory factors (e.g., Groucho-like proteins, pontin-52, ALY, etc.), that is critical for determining the outcome of cnxPg expression.

Differences between Anchored Forms of Plakoglobin and Armadillo

To further study the signaling activities of cytoplasmic catenins, it is necessary to uncouple cnxPg's ability to increase cytoplasmic levels of β-catenin from its ability to bind TCFs and other regulatory proteins. Cox *et al.* (1999) performed such an analysis in *Drosophila* using a membrane-tethered form of Armadillo. *Drosophila* appears to have a single TCF-type protein, pangolin/dTCF, which can act both positively, in conjunction with Armadillo (Brunner *et al.*, 1997; van de Wetering *et al.*, 1997), and negatively, in combination with dCBP (Waltzer and Bienz, 1998) and Groucho (Cavallo *et al.*, 1998). In the presence of the "nearly null" allele of *armadillo*, arm^{XP33}, Cox *et al.* (1999) found no evidence that anchored Armadillo altered target gene expression. However, there is an important caveat to this conclusion: tethered armadillo rescues the adhesion defects associated with *armadillo* mutations and interacts with DE-cadherin, as demonstrated by coimmunoprecipitation analyses (Cox *et al.*, 1999). It is known from studies in *Xenopus* (see Heasman *et al.*, 1994; Karnovsky and Klymkowsky, 1995; Fagotto *et al.*, 1996) and *Drosophila* (Sanson *et al.*, 1996) that binding to cadherin blocks the signaling activities of armadillo, β-catenin, and plakoglobin. Therefore, it is likely that the signaling function of tethered armadillo is inhibited by interactions with DE-cadherin (see Figure 9B). Moreover, the absence of wild-type armadillo would be expected to enhance the interaction between tethered armadillo and endogenous cadherins. In contrast, cnxPg does not efficiently reach the plasma membrane in (Figure 7, A and B; see also Merriam *et al.*, 1997), and there appears to be no substantial colocalization of cnxPg with endogenous cadherins (Figure 7, A and B). It is likely, therefore, that differences in the abilities of different anchored catenins to interact with cadherins may underlie the observed differences in their ability to activate gene expression in different systems.

It is already clear that different TCF-regulated promoters differ in their requirements for catenin cofactors. For example, the T-cell receptor α-enhancer is regulated by LEF-1 in a β-catenin–independent, ALY-dependent manner (Hsu *et al.*, 1998), whereas other TCF-regulated promoters, such as that in the cyclin D1 gene, appear to require β-catenin as a coactivator (Tetsu and McCormick, 1999) and may respond to cnxPg differently than does the OT promoter.

Although cnxPgs are artificial proteins expressed from exogenous DNAs, it is known that endogenous β-catenin and plakoglobin can form complexes with several cytoplasmic proteins. Indeed, interactions of β-catenin and plakoglobin with APC, Axin, Conductin/Axil, Presenilin, cadherins, EGFR, fascin, pontin-52, and phosphatases have all been described (see above). Binding to cadherins clearly blocks the signaling activity of plakoglobin and β-catenin (see above). It is not known, however, whether interactions between β-catenin (or plakoglobin) and other proteins inhibit the ability of the complex to bind to TCF proteins. If such interference does not occur, cytoplasmic sequestration of TCFs could play a physiological role in the regulation of

Wnt signaling. In fact, studies of endoderm formation in *Caenorhabditis elegans* indicate that nuclear levels of the TCF-like transcription factor pop-1 are reduced in response to Wnt signaling (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997) (Figure 9C). This result supports our premise that regulation of the intracellular distribution of TCFs may be involved in vertebrate Wnt signaling as well.

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