

Adenomatous Polyposis Coli Tumor Suppressor Protein Has Signaling Activity in *Xenopus laevis* Embryos Resulting in the Induction of an Ectopic Dorsoanterior Axis

Kris Vleminckx,* Ellen Wong,* Kathy Guger,* Bonnee Rubinfeld,‡ Paul Polakis,‡ and Barry M. Gumbiner*

*Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, New York 10021; and ‡Onyx Pharmaceuticals, Richmond, California 94806

Abstract. Mutations in the adenomatous polyposis coli (APC) tumor suppressor gene are linked to both familial and sporadic human colon cancer. So far, a clear biological function for the APC gene product has not been determined. We assayed the activity of APC in the early *Xenopus* embryo, which has been established as a good model for the analysis of the signaling activity of the APC-associated protein β -catenin. When expressed in the future ventral side of a four-cell embryo, full-length APC induced a secondary dorsoanterior axis and the induction of the homeobox gene *Siamois*. This is similar to the phenotype previously observed for ectopic β -catenin expression. In fact, axis induction by

APC required the availability of cytosolic β -catenin. These results indicate that APC has signaling activity in the early *Xenopus* embryo. Signaling activity resides in the central domain of the protein, a part of the molecule that is missing in most of the truncating APC mutations in colon cancer. Signaling by APC in *Xenopus* embryos is not accompanied by detectable changes in expression levels of β -catenin, indicating that it has direct positive signaling activity in addition to its role in β -catenin turnover. From these results we propose a model in which APC acts as part of the Wnt/ β -catenin signaling pathway, either upstream of, or in conjunction with, β -catenin.

THE adenomatous polyposis coli (APC)¹ gene is a tumor suppressor gene linked to familial adenomatous polyposis (FAP) and to the initiation of sporadic human colorectal cancer (9, 18, 20, 32, 40). Most FAP patients carry one allelic APC mutation in the 5' half of the coding sequence, which usually causes chain termination and results in the expression of truncated proteins. Colon polyps appear after the loss or mutation of the remaining wild-type allele, indicating that the mutant truncated APC does not act in a dominant-negative fashion by inactivating the wild type (34, 35). All mutant proteins lack the COOH-terminal half, suggesting that some growth-suppressing or growth-regulating activity is present in this part of the molecule (39).

The structure of the APC protein provides many hints about its possible cellular function. The APC gene en-

codes a 300-kD protein, consisting of 2,843 amino acids, which has several structural domains. The NH₂-terminal third contains an oligomerization domain, followed by seven armadillo repeats (imperfect repeats also found in proteins like armadillo, β -catenin, plakoglobin, and importins). The middle part of the protein contains three successive 15-amino acid (aa) repeats, followed by seven related but distinct 20-aa repeats. Both types of repeats are able to bind independently to β -catenin, a cadherin-associated protein important for intercellular adhesion (42, 43, 51). All truncated APC proteins lack most of the 20-aa repeats, but the majority of the somatic mutant APC proteins seem to have retained the 15-aa repeats and β -catenin-binding activity (39). The COOH-terminal third of the APC protein contains a basic region and has recently been shown to bind the human homologue of the *Drosophila* disc large (Dlg) tumor suppressor protein (23) and a novel protein EB1 (52). In addition, the COOH terminus can bind to microtubules and promote their assembly in vitro (28, 48).

The discovery that APC interacts with β -catenin leads to the proposal that it functions as a regulator of adhesion (14, 42, 51). β -Catenin binds to cadherins and is required for full cadherin activity. In the meantime, however, β -catenin has also been shown to have signal transduction activity. Its homologue in *Drosophila*, *armadillo*, is a segment polarity gene that acts as a component of the Wingless signal-

Address all correspondence to Barry M. Gumbiner, Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, Box 564, New York 10021. Tel.: (212) 639-6146. Fax: (212) 717-3047.

1. *Abbreviations used in this paper:* aa, amino acid; APC, adenomatous polyposis coli; hAPC, human APC; XAPC, *Xenopus* APC cDNA; XAPC FL, full-length *Xenopus* APC cDNA; Dlg, *Drosophila* disc large; EF-1, elongation factor 1; FAP, familial adenomatous polyposis; GSK-3, glycogen synthase kinase 3; dNTP, deoxyribonucleotidetriphosphate; RT, reverse transcriptase.

ing pathway (36). Furthermore, β -catenin has been found to be involved in axial patterning of early *Xenopus* embryos (8, 24). The formation of the dorsoanterior axis in *Xenopus* results from localized signaling activities in the dorsal vegetal zone (the Nieuwkoop center) of an early embryo. Injection of synthetic β -catenin mRNA in the future ventral side of an early embryo mimics the Nieuwkoop center and results in the formation of a secondary dorsal axis, manifested by the appearance of double-headed embryos. Induction of a secondary axis by β -catenin is cadherin independent, and by implication independent of cell-cell adhesion (6).

In the *Xenopus* embryo, β -catenin seems to be part of a Wnt signaling pathway, which was first identified as the Wingless pathway in *Drosophila* (21, 36). Many components of this signaling pathway were identified and epistatically ordered using genetic screens of *Drosophila* mutants that show aberrant embryonic pattern formation. These so-called segment polarity genes include *wingless* (*wg*, encoding the *Drosophila* homologue of the Wnt growth factors), *disheveled* (*dsh*, encoding a protein with unknown function), *zeste white 3/shaggy* (*zw3/shaggy*, homologue of glycogen synthase kinase 3 [GSK-3]), *armadillo* (homologue of β -catenin), and *engrailed* (a transcription factor). Wingless signaling leads to the accumulation of cytoplasmic armadillo through the inactivation of zeste white 3, a kinase that promotes the turnover of cytoplasmic armadillo protein. The Wg-induced increase in cytoplasmic armadillo protein then results in the induction of target genes, including *engrailed*. The Wingless/Wnt pathway has been remarkably well conserved in vertebrates, as evident in early *Xenopus* embryogenesis. Wnt, XDsh and β -catenin can all induce an ectopic dorsoanterior axis (8, 11, 33, 49, 50), while the Zeste White 3 homologue GSK-3 antagonizes axis formation (4, 12, 38).

Recent findings suggest that APC may also be part of the Wnt signaling pathway. APC can physically interact with both β -catenin and GSK-3, two components of the Wnt pathway (44). Also, APC seems to be a good substrate for GSK-3 in vitro, and phosphorylation of its central 20-aa repeat region promotes the binding of β -catenin. Earlier observations implicated this central region of APC in the regulation of β -catenin levels (29). However, it has not yet been shown directly that APC participates in a signaling pathway in vivo. Also, the significance of the effect of β -catenin turnover for signaling has not been determined. We used the induction of an ectopic dorsal axis in early *Xenopus* embryos as a model system to test whether APC has signaling activity and whether it influences β -catenin in vivo.

Materials and Methods

cDNA Cloning and Vector Construction

Degenerate primers flanking the β -catenin binding sites of human APC were used to generate a PCR fragment from a *Xenopus* gt-10 λ cDNA library. About 5 μ l of the library, which had a titer of 1.5×10^5 , was boiled for 5 min and directly used as a PCR template in a 100- μ l reaction consisting of 1 \times PCR buffer containing Mg^{2+} (Promega Corp., Madison, WI), dNTPs, 100 mM each, 100 pmol of each primer, and 5 U Taq polymerase (Boehringer Mannheim Biochemicals, Indianapolis, IN). The sense primer corresponded to the peptide sequence NHMDDND with a 32-fold degen-

eracy. The antisense primer, corresponding to the peptide sequence YCVEDTP, was 132-fold degenerate. Using 30 cycles of 1-min denaturing, annealing, and extension steps of 94°, 51°, and 72°C, respectively, we obtained a 750-bp fragment that when sequenced confirmed its homology to the human APC cDNA. This PCR fragment was then used to screen the *Xenopus* gt-10 λ cDNA library. We first obtained clone 6A, encompassing the NH₂-terminal third (including 100 bp of untranslated region) of the *Xenopus* APC cDNA and clone 3bF1 containing the middle part of *Xenopus* APC cDNA. Using the 400 bp of the 3' end of 3bF1 as a probe, we rescreened the library and obtained clone 40.2, which encompassed 3bF1 but extended 450 bp further to the 3' end, and a clone 36.2 of ~3 kb, partially overlapping with 3bF1 and 40.2 but extending 2.2 kb further to the COOH terminus, still 150 bp short of the stop codon. A final screen of the library with a 600-bp probe obtained from the 3' end of clone 36.2 ultimately generated clone 111 containing the complete COOH-terminal third, including the stop codon and 150 bp of 3' untranslated region.

The full-length *Xenopus* APC cDNA (XAPC FL) and cDNAs encoding APC fragments (XAPC 1, XAPC 4, and XAPC 5) were constructed by ligating clones 6A, 40.2, and 111 via their EcoRI restriction sites. All fragments were modified at their 5' end to introduce a start codon contained in an NcoI site and NH₂-terminally linked to six myc-epitope tags in the vector pCS2+MT (45, 53), which was used for in vitro transcription.

The full-length human APC (hAPC) cDNA and the cDNA fragments hAPC 21 and hAPC 25, all having an altered 5' end containing a start codon in the NcoI restriction site, were isolated out of a baculovirus transfer vector (42) with the appropriate restriction enzymes and inserted into the vectors pCS2+ or alternatively into pCS2+MT, for which the APC fragments were linked 5' with a myc-epitope tag.

Immunoprecipitations and Western Blotting

Expression of endogenous *Xenopus* APC and its association with β -catenin and cadherins were analyzed by immunoprecipitation and Western blotting. About 10 embryos were lysed in 250 μ l NP-40 lysis buffer (0.5% NP-40, 150 mM NaCl, 2 mM EDTA, 10 mM HEPES-NaOH, pH 7.4, 0.02% NaN₃) and centrifuged for 10 min, and the supernatant was incubated either for 3 h with anti- β -catenin pAb (serum, diluted 1:500) or overnight with anti-APC3 pAb (serum, diluted 1:250). Immunocomplexes were precipitated for 1 h with protein A-Sepharose, washed five times with NP-40 lysis buffer, and eluted in SDS sample buffer. For the detection of APC, samples were separated on 3% agarose gels that were transferred to nitrocellulose by capillary blotting as described (47). Blots were blocked in PBS supplemented with 5% dry milk and 0.2% Triton X-100 and incubated in the same buffer overnight at 4°C with anti-APC3 pAb (diluted 1:5,000) followed by HRP-conjugated anti-rabbit IgG (Bio Rad Laboratories, Melville, NY; diluted 1:3,000) for detection of *Xenopus* APC proteins. Blots were washed and developed with enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL). For the detection of β -catenin and cadherins, samples were loaded on 7% SDS polyacrylamide gel, separated, and blotted to nitrocellulose. For detection of β -catenin, blots were incubated for 1 h with anti- β -catenin pAb (serum, diluted 1:5,000) followed by HRP-conjugated anti-rabbit IgG (Bio Rad Laboratories; diluted 1:3,000). Cadherins were detected either with a mixture of C-cadherin-specific mAbs 6B6 and 5G5 (hybridoma supernatant, 1:3 diluted) or with a mixture of E-cadherin-specific mAbs 19A2 and 5D3 (hybridoma supernatant, 1:3 diluted), followed by HRP-conjugated anti-mouse IgG (Bio Rad Laboratories; diluted 1:3,000).

Expression of exogenous APC fragments was also analyzed by Western blotting. Typically, 5–10 embryos were lysed in 250 μ l NP-40 lysis buffer. Lysates were supplemented with an equal volume of 2 \times SDS sample buffer for analysis of total lysates. Samples were loaded on 3% agarose gels, separated, and immunoblotted as described above. Blots were incubated for 1 h at room temperature with anti-myc tag mAb 9E10 (5) (ascites, diluted 1:1,000) followed by HRP-conjugated anti-mouse IgG (Bio Rad Laboratories; diluted 1:3,000) for detection of myc-tagged APC proteins.

Reverse Transcriptase-PCR Assay

Reverse transcriptase (RT)-PCR was performed as described (3, 45). Typically two embryos or four animal caps were homogenized in 100 μ l lysis buffer (0.5% SDS, 5 mM EDTA, 50 mM NaCl, and 50 mM Tris, pH 7.5) containing 250 μ g/ml proteinase K (Boehringer Mannheim Biochemicals), incubated 30 min at 45°C, and extracted with phenol/chloroform. After the addition of 10 μ g clean yeast tRNA as carrier, nucleic acids were

precipitated with 1 M NH₄OAc and EtOH. DNA was removed from the samples by RNase-free DNase (Boehringer Mannheim Biochemicals) at a concentration of 2 U/25 ml in DNase buffer (10 mM NaCl, 6 mM MgCl₂, 100 mM CaCl₂, 40 mM Tris, pH 7.9) containing 1 mM DTT and 15 U RNasin (Promega). After a phenol/chloroform extraction, RNA was precipitated with NH₄OAc and EtOH. Half of the RNA samples were then denatured at 65°C, and hybridized with random hexamers (Boehringer Mannheim Biochemicals), and cDNA was made using the Superscript Preamplification System (GIBCO BRL, Gaithersburg, MD). A 20- μ l reaction mixture consisted of 1 \times first strand buffer (GIBCO BRL), a dNTP mix of 500 mM of each nucleotide, 100 mM DTT, 15 U RNasin (Promega), and 25 U RT (GIBCO BRL). Reactions were incubated for 30 min at 42°C. One fifth of the cDNA samples was then used for PCR amplification using either primers for the Siamois cDNA or the ubiquitous expressed elongation factor 1 (EF-1) cDNA, using a hot start of 93°C for 2.5 min and 25 cycles of 93°C for 30 s, 55°C for 1 min, and 72°C for 30 s. The reaction was terminated with an incubation of 5 min at 72°C. A 25- μ l reaction consisted of 1 \times PCR buffer with Mg²⁺ (Boehringer Mannheim Biochemicals), dNTPs at 1 mM each, 1 μ Ci of [³²P]dCTP (Amersham Corp.), and 1.25 U of Taq DNA polymerase (Boehringer Mannheim Biochemicals). The sense and antisense primers for Siamois detection were TTGCTCTACCGCACTGA and TCCTGTTGACTGCAGACT, respectively, which should generate a 307-bp fragment. However, we always obtained a double band, the higher of which was always proportional to the 307-bp band. The sense and antisense primers for EF-1 detection were CAGATTGGTGCTGGATATGC and ACTGCCTTGATGACTCCTAG, respectively, generating a 268-bp fragment. PCR samples were separated on a 5% acrylamide gel and run in 0.5 \times Tris borate EDTA buffer, and PCR fragments were visualized by autoradiography.

RNA and Protein Injections

All APC constructs were linearized and capped RNAs were synthesized in vitro using SP6 RNA polymerase (Promega). mRNAs were dissolved in diethyl pyrocarbonate-treated water at a concentration of 0.125–0.5 ng/nl and injected in a volume of ~15 nl into the equatorial region of a single prospective ventral (or two dorsal) blastomere of a four-cell stage embryo. During injection, embryos were kept in a solution of 1 \times Marc's Modified Ringer solution (MMR; 100 mM NaCl, 2 mM KCl, 5 mM Hepes-NaOH, pH 7.8, 2 mM CaCl₂, and 1 mM MgSO₄) supplemented with 6% ficoll to improve healing, 100 U/ml penicillin, and 100 μ g/ml streptomycin. After injection, embryos were further incubated in 0.1 \times MMR supplemented with antibiotics and scored at stage 16–18 for duplicated axis, visible by the presence of two darkly pigmented neural folds. Staging was according to Nieuwkoop and Faber (31).

Recombinant APC proteins, eluted in a buffer containing 20 mM Tris, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1 mM DTT, 10 μ g/ml pepstatin, 10 μ g/ml leupeptin, 0.5 mM Pefabloc, and 1 μ M aprotinin, were concentrated to 2–4 mg/ml (except for APC 22 for which the final concentration was 0.2

mg/ml) using amicon filters, and then injected as described above for the synthetic mRNA.

Results

Xenopus APC Is Expressed in Early Embryos and Is Associated with β -Catenin But Not with Cadherins

To determine whether APC is expressed in early *Xenopus* embryos, the APC protein was detected by immunoprecipitation of samples of unfertilized eggs and early embryos at different developmental stages. Immunocomplexes, separated on a polyacrylamide gel, were immunoblotted with anti-APC 3 antibodies, revealing a 300-kD band at all stages analyzed (Fig. 1 A). Signals for endogenous APC were increased at least twofold when the embryos were lysed in buffer containing deoxycholate (data not shown). To determine whether *Xenopus* APC interacts with β -catenin or cadherins, samples from stage 11 embryos were immunoprecipitated with either anti-APC 3 or anti- β -catenin antibodies, and subsequently immunoblotted with either anti-APC 3, anti- β -catenin, anti-E-cadherin, or anti-C-cadherin antibodies. APC coimmunoprecipitated with β -catenin (Fig. 1 B). However, although cadherins coimmunoprecipitated with β -catenin, they did not coimmunoprecipitate with APC (Fig. 1 B). Therefore, in early embryos, *Xenopus* APC is apparently complexed with β -catenin but not with cadherins, just as it is in mammalian cell lines (16, 43).

Cloning of *Xenopus* APC cDNA

We cloned and sequenced APC from a stage 17 *Xenopus* gt-10 λ cDNA library. The *Xenopus* APC cDNA encoded a protein with an estimated molecular mass of 311 kD that showed 75% sequence similarity with its human counterpart. High sequence conservation was observed in the currently described structural motifs, including the oligomerization domain (85% similarity), the armadillo repeats (97%), the 15-aa repeats (80 and 100%), and the 20-aa repeats (90–100%) (Fig. 2). Only two of the three 15-aa re-

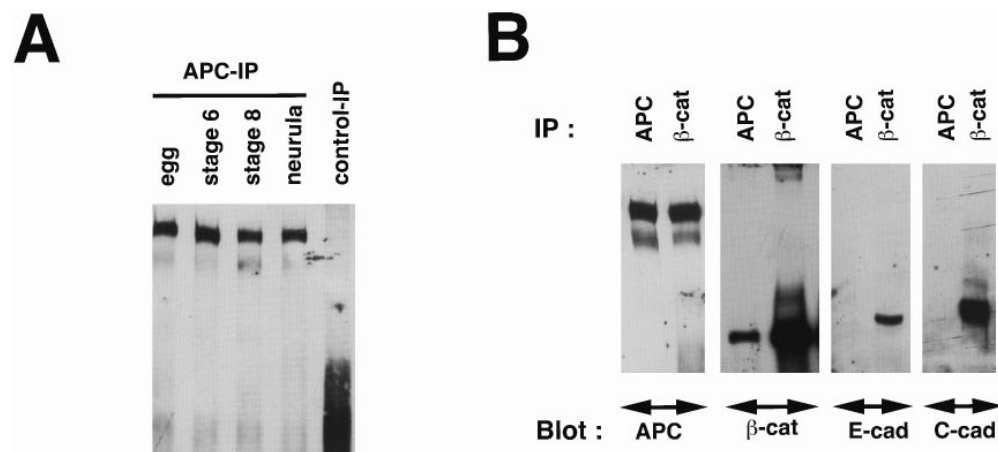


Figure 1. *Xenopus* APC is expressed throughout early embryogenesis and is associated with β -catenin but not with cadherins. (A) Immunoprecipitation and immunoblotting of endogenous *Xenopus* APC protein by anti-APC 3 antibodies. *Xenopus* APC is present in the egg and at least until neurula stage. (B) Interaction of APC with β -catenin but not with cadherins. Stage 11 embryos were lysed in NP-40 buffer and immunoprecipitated (IP) with either APC 3 or β -catenin antibodies. Immunocomplexes were blotted (Blot) for either APC, β -catenin, E-cadherin, or C-cadherin.

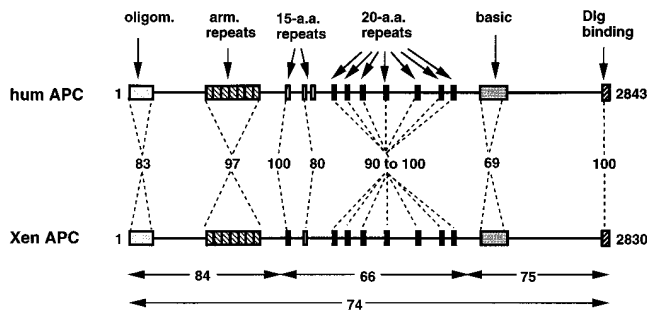


Figure 2. Comparison of *Xenopus* and human APC sequences. Linear representation of the amino acid sequence similarities between human and *Xenopus* APC. Numbers indicate the similarity of the sequences that were aligned by the Clustal method (PAM matrix 250, MegAlign; DNASTAR, Inc., Madison, WI). The described structural domains including the oligomerization domain (*oligom.*), armadillo repeats (*arm. repeats*), 15-aa repeats, 20-aa repeats, basic domain, and Dlg binding site are shown. Numbers beneath the sequences indicate the sequence similarity of the whole protein, or of the NH₂-terminal 1,000 amino acids, the middle 1,000 amino acids, and the COOH-terminal 850 amino acids, respectively. The *Xenopus* APC sequence data are available from EMBL/GenBank/DBJ under accession number U64442.

peaks found in human APC, which were first identified as the β -catenin binding sites, are present. In these, the consensus sequence EX(D/E)XPXNYSX(K/R)YX(D/E)E was conserved (51). All seven 20-aa repeats found in human

APC, which can also mediate β -catenin binding, were conserved and showed the (E/D)XXPXX(F/Y)S consensus sequence (43). The basic domain was less conserved (67%). The COOH-terminal 15 aa, which have recently been shown to interact with the human homologue of the *Drosophila* Dlg tumor suppressor protein (23), were 100% conserved. We conclude that we have indeed cloned the *Xenopus* homologue of the human APC tumor suppressor gene.

Full-Length *Xenopus* APC Induces a Secondary Dorsoanterior Axis

For expression experiments, the partial *Xenopus* APC cDNA clones were subcloned separately or linked together to form a cDNA encoding the entire open reading frame (Fig. 3 A). To validate the expression in embryos, full-length XAPC and all partial constructs had an NH₂-terminal myc tag. Synthetic mRNA was injected into four-cell stage embryos. All fragments were very well expressed as analyzed by immunoblotting with antibodies against the myc-epitope tag (Fig. 3 B; not shown for XAPC 5). The lower molecular weight bands detected during expression of full-length XAPC FL seem to be degradation products generated during sample preparation, because extraction of endogenous *Xenopus* APC from embryos always results in similar prominent bands of lower molecular weight when blotted with anti-APC 3 antibodies (data not shown). Thus, the exogenous APC mRNA is expressed and its product behaves like endogenous APC.

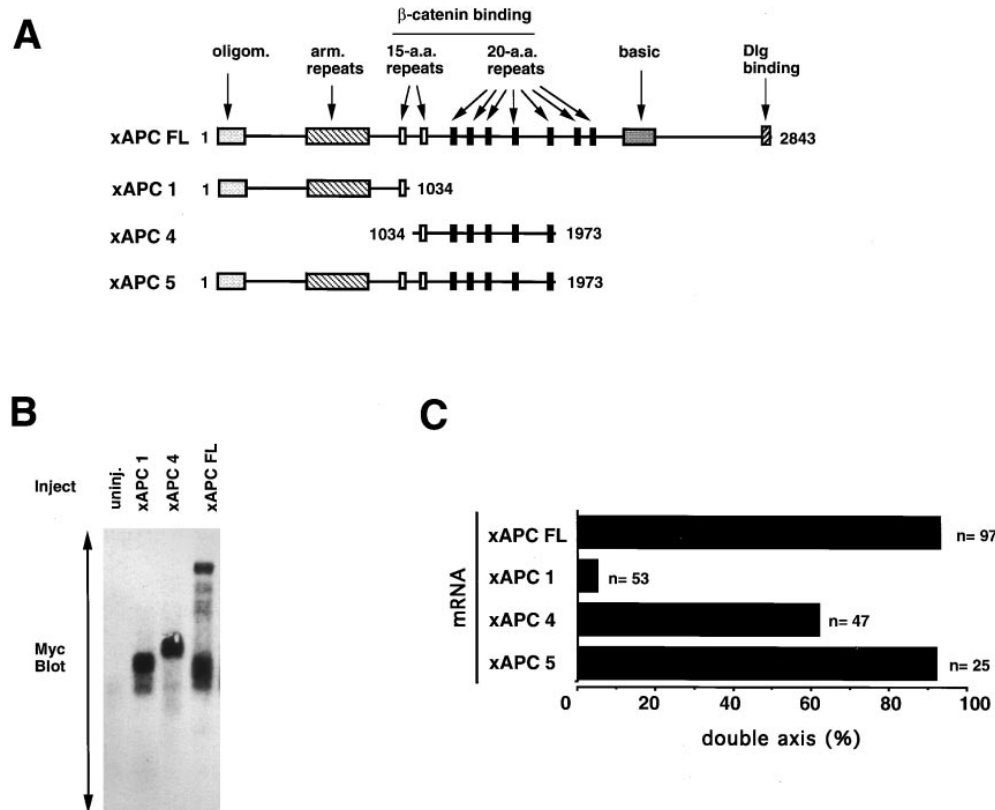


Figure 3. Full-length *Xenopus* APC or fragments containing the central domain induce an ectopic dorsoanterior axis. (A) Linear representation of the full-length *Xenopus* APC (*xAPC FL*) and the various fragments used (*xAPC 1, 4, and 5*). The amino acid positions of each fragment are indicated by the flanking numbers. The motifs, identified in the human APC and conserved in the *Xenopus* APC cDNA, are shown on top, including the oligomerization domain (*oligom.*), armadillo repeats (*arm. repeats*), 15- and 20-aa repeats, basic domain, and Dlg binding site. (B) Expression levels of APC fragments resulting from the injection of mRNA of XAPC 1, XAPC 4, and XAPC FL, respectively. Expression was detected by immunoblotting using the anti-myc tag mAb. (C) Bar graph summarizing the frequency of axis induction by the different *Xenopus*

APC fragments, expressed from injected synthetic mRNA in four-cell stage embryos. Secondary axis formation is plotted as a percentage, and the total number of embryos analyzed is indicated to the right of each bar (n).

Injection of XAPC FL mRNA into the ventral side of four-cell embryos very efficiently induced the formation of secondary dorsoanterior axes (Fig. 3 C). This often resulted in the generation of Siamese twins connected by trunk and tail. The phenotype was most clear at the neurula stage (stage 14–15) when the nascent neural tubes are visible as darkly pigmented lines. Accordingly, this stage was used for scoring the frequency of axis duplication. Axis induction occurred >90% of the time with 5–10 ng of XAPC FL mRNA (Fig. 3 C) and resulted in the development of double-headed tadpoles. Ectopic dorsal axes were also induced by expression of the fragments XAPC 4 and, even more efficiently, XAPC 5. These findings indicate that APC is able to induce an ectopic dorsoanterior axis, and consequently has signal transduction activity. Moreover, the central part of the APC is the minimal requirement for axis induction.

The NH₂-terminal fragment XAPC 1, which is similar to a truncated human APC molecule frequently found in colon cancer, caused only a very low background of secondary axis induction (6%), and, when observed, the extra axes were always very short and incomplete. The truncated XAPC 1 fragment still contains a β -catenin binding site and actually binds β -catenin much more efficiently than the central part of the APC protein that is required and sufficient for axis induction (data not shown). This excludes a model in which axis induction by APC results from simply binding up β -catenin.

Expression of the NH₂-terminal β -catenin binding *Xenopus* APC fragment in the future dorsal side of a four-cell stage embryo did not interfere with the formation of the endogenous dorsal axis (data not shown), and therefore this fragment does not inhibit signal transduction processes involved in axis determination. Furthermore, NH₂-terminal APC fragments that lack the central domain were unable to antagonize the axis-inducing APC fragments when coinjected (data not shown), indicating that they have no dominant-negative properties.

The Central Domain of Human APC Also Has Signaling Activity

Since human APC has been well characterized in terms of the domains participating in various molecular interactions and truncations associated with colon carcinogenesis, we also injected human APC fragments, either as recombinant protein or as synthetic mRNA (Fig. 4, A and B). Unfortunately, we were unable to get efficient expression of the full-length human APC (Fig. 4 D). However, fragments representing the central part of the molecule induced an ectopic dorsoanterior axis very efficiently. 80–90% of the embryos injected with hAPC 2 or hAPC 25 in the ventral side showed a second axis (Fig. 4 B), many of which had complete new head structures, including a cement gland and pigmented eyes (Fig. 4 C). Formation of a weak partial secondary axis was observed in only 7% of the em-

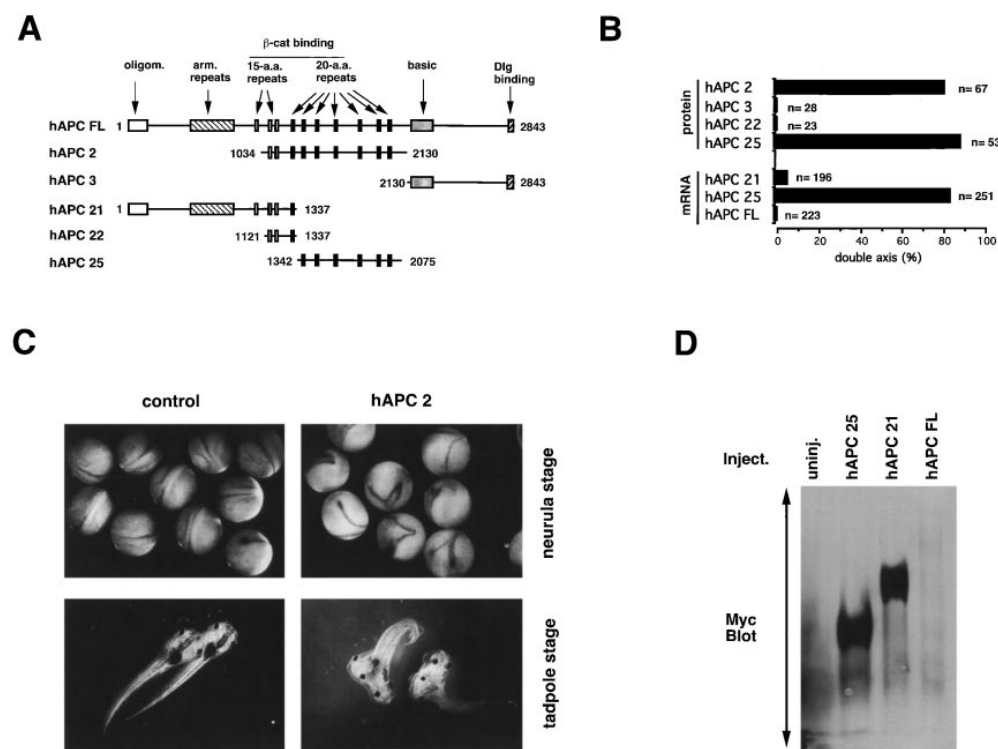


Figure 4. Human APC fragments induce an ectopic dorsoanterior axis in early *Xenopus* embryos. (A) Linear representation of the full-length human APC (hAPC FL) and the various fragments used (hAPC 2, 3, 21, 22, and 25). The amino acid positions of each fragment are indicated by the flanking numbers. Several known motifs are shown on top, including the oligomerization domain (oligom.), armadillo repeats (arm. repeats), the 15- and 20-aa repeats (both known to bind β -catenin), basic domain, and Dlg binding site. (B) Bar graph summarizing the frequency of axis induction by the different APC fragments, either injected as recombinant protein (upper) or expressed from synthetic mRNA (lower) in four-cell stage embryos. Secondary axis formation is plotted as a percent-

age, and the total number of embryos (*n*) analyzed is indicated to the right of each bar. (C) Micrographs showing the double axis phenotype in embryos injected with recombinant hAPC 2 protein. Embryos are shown at stage 14 (neurula stage), where the forming neural tubes are visible as darkly pigmented lines, and at the tadpole stage, showing embryos with two complete heads, including two eye pairs. (D) Expression levels of APC fragments resulting from the injection of mRNA for hAPC 25, hAPC 21, and hAPC FL, respectively. APC fragments were detected by immunoblots using the anti-myc tag mAb.

bryos injected with mRNA encoding the NH₂-terminal fragment hAPC 21, and the few embryos scored as positive had only very short and incomplete secondary axes. A COOH-terminal fragment hAPC3 was also unable to induce an ectopic dorsoanterior axis. Therefore, similar to the results with *Xenopus* APC, the central domain of the human APC protein has signal transduction activity in the early *Xenopus* embryo. Fragments that lack the central domain, like the NH₂-terminal hAPC 21, which corresponds to a mutant APC frequently observed in colon cancer, do not show this signal transduction activity.

APC Induces the Expression of a Marker of the Nieuwkoop Center, the Homeobox Gene *Siamois*

Dorsoanterior axis induction by APC is very similar to the phenotype observed for β -catenin mRNA injections, and therefore they may act at a similar site in the embryo. β -Catenin participates in the early signaling events in a region called the Nieuwkoop center (11). Activation of the Nieuwkoop center by β -catenin and Wnt growth factors induces the expression of the homeobox protein *Siamois*, which itself causes axis duplication when injected in the ventral side of an embryo (22). β -Catenin signaling activity seems to directly induce *Siamois* (Nelson, R., personal communication). Induction of *Siamois* is selective, however, because it is not induced by other factors that induce markers of a later signaling center, the Spemann organizer, such as noggin, activin, and Vg-1 (7). To determine whether APC, like β -catenin and Wnt, can act in the Nieuwkoop center, we determined whether ectopic expression of *Xenopus* APC or human APC could induce *Siamois* expression. APC fragments were injected into the animal pole of four-cell stage embryos. At stage 8, animal caps were dissected and cultured as explants to remove the endogenous Nieuwkoop center in which *Siamois* is normally expressed. Expression of *Siamois* in animal caps was assayed by RT-PCR. Full-length *Xenopus* APC clearly induced *Siamois* expression (Fig. 5 A). In contrast, the XAPC 1 fragment, which corresponds to a truncated APC mutant that has no

major axis-inducing activity, did not induce *Siamois* expression. Injection of the human hAPC 25 mRNA also induced *Siamois* expression in the animal caps in a dose-dependent manner (Fig. 5 B). Therefore, like β -catenin and Wnt, the axis-inducing APC fragments mimic the Nieuwkoop center in the early embryo, suggesting that APC and β -catenin act in the same signaling pathway.

Role of β -Catenin in Axis Induction by APC

To determine whether induction of a secondary axis by APC actually depends on β -catenin, we used overexpression of C-cadherin as a competitive inhibitor. C-Cadherin is the major cadherin expressed in the early embryo, and it binds β -catenin with high affinity. Overexpression of C-cadherin in early embryos depletes the blastomeres of free, non-membrane-bound β -catenin and inhibits its signaling activity (6). C-Cadherin overexpression in oocytes or in the dorsal vegetal region of embryos results in completely ventralized embryos (6, 13), and, when coexpressed with β -catenin, C-cadherin strongly antagonizes the induction of a secondary axis (6). Therefore, to test the requirement of β -catenin for APC signaling, we injected 4 ng of hAPC 25 or XAPC FL mRNA either alone or in the presence of 2–5 ng of C-cadherin mRNA. Coexpression of C-cadherin completely abolished axis duplication by hAPC 25 and XAPC FL mRNA (Fig. 6). Therefore, free cytosolic β -catenin seems to be required for hAPC 25 and XAPC FL to induce axis duplication. The simplest interpretation of this epistasis experiment is either that APC and β -catenin act together as a complex or that β -catenin acts downstream of APC. However, a more complex model in which β -catenin influences the active state of APC cannot be ruled out.

Levels of β -Catenin Are Not Influenced by Overexpressed APC

Certain colon cell lines that express mutant APC contain high levels of β -catenin. Transfection of these cell lines with full-length human APC, or fragments containing the

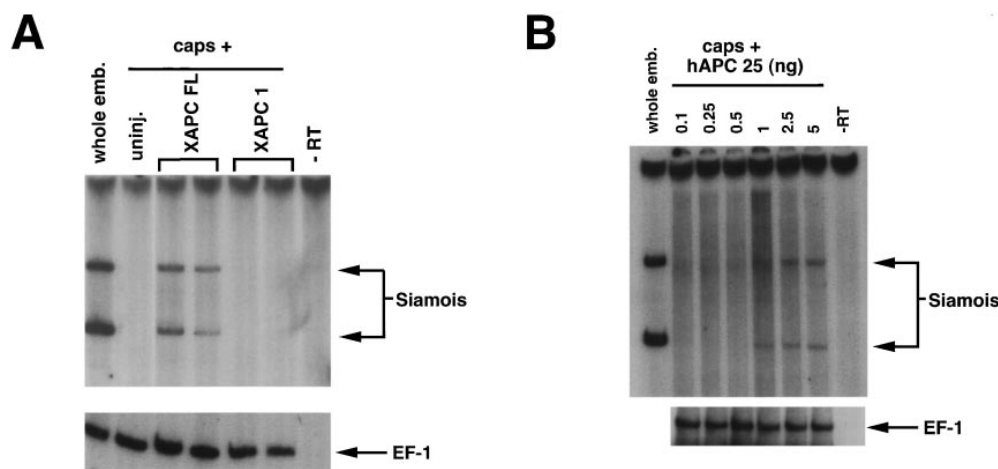


Figure 5. APC induces the expression of the *Hox* gene, *Siamois*. (A) *Siamois* expression is induced only by APC fragments with axis-inducing activity. RNA was extracted from animal caps injected with synthetic RNA encoding either *Xenopus* full-length APC (*XAPC FL*) or the NH₂-terminal third of the APC cDNA (*XAPC 1*) and analyzed for *Siamois* expression by RT-PCR. The ubiquitously expressed elongation factor 1 (*EF-1*) was also included as a loading control. As a positive control, RT-PCR reactions from whole

embryos were loaded. To rule out the possible contamination of genomic DNA, a sample that was not treated with reverse transcriptase was included (-RT). (B) RT-PCR analysis of the expression of *Siamois* in animal caps injected with increasing amounts of synthetic RNA encoding human hAPC 25 mRNA. Whole embryo and uninjected animal caps were loaded as positive and negative controls, respectively.

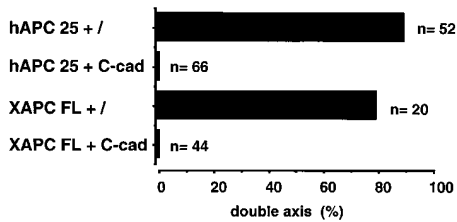


Figure 6. The induction of an ectopic dorsal axis by APC requires free cytosolic β -catenin. APC signaling is inhibited by coexpressed C-cadherin, which sequesters β -catenin at the membrane. Bar graph summarizing the frequency of axis induction of embryos injected at the four-cell stage at their ventral site with 4 ng of hAPC 25 and XAPC FL mRNA, injected either alone or in combination with 2 ng of C-cadherin mRNA.

central 20-aa repeats, significantly reduces total β -catenin levels (29). From these results it has been proposed that APC is able to increase the turnover of β -catenin, thereby regulating its cytoplasmic levels and its availability to interact with other proteins. Interestingly, the same or similar APC fragments that reduce β -catenin levels in these colon cancer cell lines are able to induce an ectopic dorsal body axis in *Xenopus*. This seems contradictory, since ectopic APC expression causes a phenotype resembling the one caused by overexpressed β -catenin rather than reduced levels of β -catenin. Therefore, we asked whether full-length *Xenopus* XAPC and the active human hAPC 25 fragment were able to influence β -catenin levels in early *Xenopus* embryos.

To achieve uniform expression of exogenous APC, APC mRNA was injected into the animal pole of all four blastomeres of the four-cell embryo, and animal caps were explanted at stage 8. Expression levels of β -catenin in these animal caps were determined by immunoblotting. For a loading control, C-cadherin was also detected. As shown in Fig. 7 A, β -catenin levels were largely unchanged in the animal caps expressing XAPC FL. Similarly, no changes in β -catenin levels were observed after expression of the axis-inducing human APC fragment hAPC 25 (Fig. 7 A). We also examined the effects of APC expression on exogenously expressed β -catenin, to be sure that the levels of β -catenin were being measured in the same cells that expressed exogenous APC. Myc-tagged β -catenin mRNA was coinjected with the full-length *Xenopus* APC mRNA or with hAPC 25 mRNA, and embryos were lysed after 2.5 h (stage 7) or 4 h (stage 9). The levels of exogenously expressed β -catenin were determined by immunoblotting using anti-myc antibodies. As shown in Fig. 7 B, expression levels of β -catenin were not significantly influenced by XAPC FL or by hAPC 25. Apparently, the dorsoanterior axis induction by APC is not associated with any major changes in expression levels of the endogenous β -catenin.

It is formally possible that APC acts through regulating the levels of plakoglobin, a protein that is very similar to β -catenin. Like β -catenin, plakoglobin is found to associate with both cadherins and APC and is also able to induce an ectopic dorsoanterior axis in *Xenopus* (19). Therefore, we examined endogenous plakoglobin levels in animal caps injected with XAPC FL mRNA. No differences between the levels of plakoglobin in uninjected or XAPC

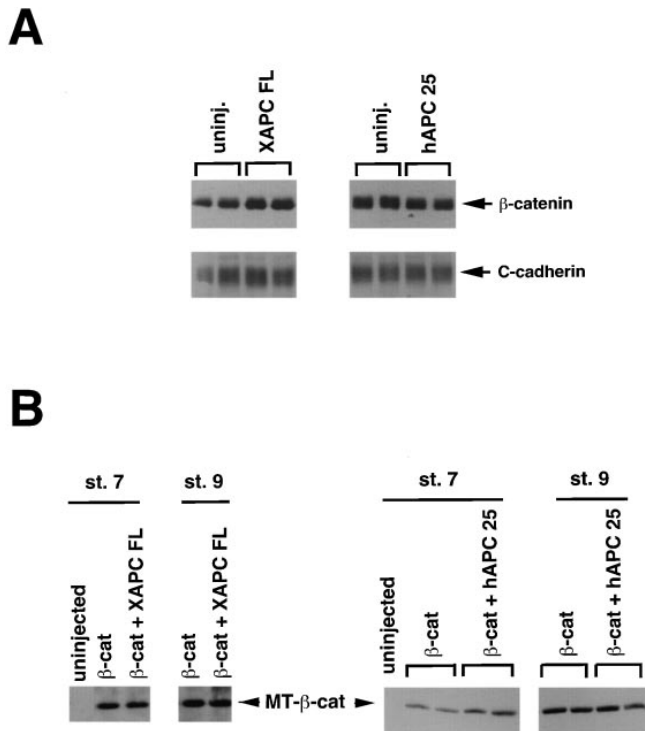


Figure 7. Overexpression of APC in *Xenopus* does not cause major changes in levels of β -catenin. (A) Levels of endogenous β -catenin in animal caps that were isolated from embryos injected four times in their animal pole with either hAPC 25 or XAPC FL. β -Catenin expression was detected by immunoblotting. For a loading control, samples were blotted for C-cadherin. (B) Expression levels of myc-tagged β -catenin from exogenous mRNA in stage 7 and stage 9 embryos that were injected earlier at four-cell stage either with 1 ng of myc-tagged β -catenin mRNA alone or in combination with 4 ng of hAPC 25 or XAPC FL mRNA.

FL-expressing animal caps were observed (data not shown). Thus, it is unlikely that dorsoanterior axis induction by APC is mediated by changes in plakoglobin levels.

Discussion

Our findings implicate the APC tumor suppressor gene in the process of axial patterning in *Xenopus*. Since induction of the dorsoanterior axis involves the activities of embryonic signaling centers (or organizers), these findings demonstrate that APC has signal transduction activity. APC seems to act in the same signaling pathway as β -catenin. They have similar characteristics, including induction of the homeobox protein Siamois. Furthermore, APC signaling is strongly dependent on the availability of a free cytosolic pool of β -catenin, which by itself has axis-inducing activity. Moreover, APC or APC/ β -catenin complexes seem to have direct positive signaling activity, since APC does not act indirectly simply by binding up β -catenin or by changing the levels of β -catenin. We propose, therefore, that axis induction by APC is due to its role in a signal transduction process in which β -catenin has been strongly implicated.

The induction of a dorsoanterior axis in *Xenopus* results

from localized signaling activity in the early embryo. After fertilization of the egg, an early dorsalizing zone, called the Nieuwkoop center, becomes localized and activated in a vegetal region of the embryo. Later in development, after the onset of transcription, the Nieuwkoop center induces overlying mesoderm to form a second dorsalizing center, called the Spemann organizer. In the last decade, several factors have been identified that can mimic or inhibit either of the two dorsalizing centers. These factors include secreted signaling molecules, cytoplasmic factors that act as signal transducers, and transcription factors. Frequently, these different factors are components of a unique signaling pathway; e.g., Wnt, Dsh, Gsk-3, and β -catenin are all part of the Wnt pathway. Here we demonstrate that APC is able to mimic a dorsalizing center, probably the Nieuwkoop center, suggesting that it also has signal transducing activity.

Comparison of the axis-inducing activities of the different *Xenopus* and human APC fragments indicates that the central domain of the APC protein is both sufficient and essential for ectopic axis induction. Truncated APC mutants that lack this central domain of the protein are ineffective in dorsoanterior axis induction, indicating that they lack the signaling activity. The central domain of APC is missing in the majority of the truncating APC mutations expressed in tumors from FAP patients and in spontaneous colon tumors, suggesting that some growth-suppressive or growth-regulatory activity is present in this region (39). We hypothesize that the loss of growth-regulatory activity results from the loss of the signaling activity present in the central part of APC.

Polyp formation in the colon occurs after a loss of heterozygosity or a mutation of the remaining APC allele, indicating that the truncated APC mutants are not dominant-negative proteins (34, 35). Consistent with the data from colon carcinogenesis, the COOH-terminally truncated APC mutants have no dominant-negative properties in the *Xenopus* embryo, since they cannot interfere with the formation of the endogenous dorsal axis and cannot antagonize the effects of axis-inducing APC fragments when coinjected (data not shown).

The cellular function of the APC protein was a complete mystery until the discovery that it binds catenins, proteins associated with cadherin adhesion molecules. The association of APC with catenins initially led to the proposal that APC has a role in regulating the activity of the catenin/cadherin complex and thus intercellular adhesion (14, 42, 51). This seemed to have implications for colon carcinogenesis because changes in cell adhesion could affect processes like cell migration and shedding, which could be important for the development of colon polyps. Also, it has been proposed that APC is involved in cell migration (30). However, the discovery that β -catenin and plakoglobin have signal transducing activity in *Xenopus* embryos (8, 13, 19), independent of their role in cell adhesion, raised the possibility that the observed APC/catenin association reflects a role for APC in signal transduction. Although a role for APC in cell adhesion cannot be ruled out, our data demonstrate that the cellular function of APC is certainly not limited to the regulation of the adhesiveness and migration of cells. Axis induction in *Xenopus* by APC is identical to the phenotype resulting from overexpressed β -cate-

nin, and therefore involves a related signal transduction activity.

APC may be involved in a Wnt-like signaling pathway. Ectopically expressed β -catenin is known to mimic the Nieuwkoop center (11). Similarly, APC induces expression of Siamois, a marker for this Nieuwkoop center (22). This signaling activity is shared by several components of the Wnt signaling pathway, including Wnts themselves and a kinase-mutant form of GSK-3. Our findings suggest that APC and β -catenin act at the same place in this signaling pathway. Not only do they form complexes in the early *Xenopus* embryo, but axis induction by APC also depends on the availability of free cytosolic β -catenin. Overexpression of C-cadherin, which sequesters the cytosolic β -catenin to the membrane, strongly inhibits axis induction by APC. In fact, C-cadherin injection can inhibit axis formation induced by the different components of the Wnt/Wg signaling pathway that act upstream of β -catenin, like XWnt 8 and kinase-defective GSK-3, but it is not able to suppress axis formation by factors that act independently from the Wnt signaling pathway (e.g., Vg-1, activin, and noggin) or that act downstream of β -catenin (e.g., Siamois) (7). The inhibition of APC signaling by depletion of cytosolic β -catenin suggests that APC acts either upstream of β -catenin or that APC and β -catenin act together as a complex.

Our findings do not directly address whether APC is a component of a Wnt pathway rather than, for example, an independent or parallel regulator of β -catenin. However, there are biochemical data showing that APC can physically interact with another component of the Wnt/Wg pathway, GSK-3 (homologue of the *Drosophila* zw-3) (44). Moreover, APC seems to be a good substrate for GSK-3 in vitro. Together, these links of APC to both upstream and downstream components of the Wnt pathway suggest that it is directly involved in the signal transduction mechanism. Nevertheless, proof that APC is indeed part of the Wnt/Wg pathway will require a demonstration that the loss or inhibition of its function blocks Wnt signaling.

APC has been reported to be able to reduce β -catenin expression levels in certain colon cancer cell lines by enhancing its turnover (29). Interestingly, the activation of the wingless pathway in *Drosophila* seems to result in increased stability of Armadillo protein (41, 54), and overexpression of a kinase-dead GSK-3 in *Xenopus* also results in increased stability of β -catenin (56). From this finding, it has been speculated that APC, through its proposed ability to regulate the stability of β -catenin, is a negative regulator in the Wnt/Wg pathway (25, 37). However, increased turnover of β -catenin by APC in the colon cancer cell lines was not related to any observable cellular function. Furthermore, induction of apoptosis by APC introduction in other colon cancer cells was not accompanied by reduction of β -catenin levels (27). Similarly, we find that the APC signaling activity in early *Xenopus* embryos, which is manifested by dorsal axis induction, is apparently not due to its regulation of β -catenin levels. First, the enhanced turnover of β -catenin by APC or the central repeats in colon cancer cell lines are inconsistent with our findings that the very same APC molecules stimulate axis induction, a process associated with increased levels of β -catenin. Second, we could not detect changes in the lev-

els of either endogenous β -catenin (or plakoglobin) or β -catenin expressed from injected mRNA, in response to expression of either full-length *Xenopus* APC or the axis-inducing central fragment of the human APC. Our findings in *Xenopus* embryos suggest that APC, or APC/ β -catenin complexes, have signaling activity on their own, independent from the regulation of β -catenin levels. It is possible that the increased turnover of β -catenin by APC, which is observed in colon cancer cell lines, is part of a different signaling mechanism, or is the result of a feedback loop that turns off the signaling by APC and β -catenin. Our results also suggest that APC is an activating, rather than an inhibiting, component of the Wnt/Wg pathway. More research is needed to clarify the exact function and activity of APC in this pathway.

Both overexpressed β -catenin and plakoglobin are known to localize in the nuclei of injected *Xenopus* blastomeres (8, 19). Endogenous β -catenin also has been found in the nuclei of blastomeres at the dorsal side of both *Xenopus* and Zebrafish embryos (46, 56). β -Catenin lacks the classical nuclear localization sequence needed for nuclear import. However, members of the lymphoid enhancer-1/Tcf family of transcription factors have recently been identified as proteins that bind to β -catenin and can, under certain conditions, carry β -catenin to the nucleus (2, 15, 26). We have not yet been able to detect major changes in the subcellular localization of endogenous β -catenin in blastomeres overexpressing ectopic full-length *Xenopus* APC (data not shown). This is, however, most likely due to lack of sensitivity since it was not easy to detect nuclear localization of endogenous β -catenin either in the natural or Wnt-induced dorsal side of *Xenopus* embryos (8, 56).

Using axis formation in *Xenopus* embryogenesis as a model system to investigate signaling pathways, we have uncovered a signaling function for APC that might be relevant for understanding its role in colon carcinogenesis. One role for APC signaling in oncogenesis might be the regulation of cell proliferation or sensitivity to transformation. APC has been shown to alter the transformation properties, decrease the growth rate or induce apoptosis in colon carcinoma cells (10, 27), and block the cell cycle progression from G0/G1 to S phase in fibroblasts (1), possibly by regulating the activity of cyclin-dependent kinase complexes. β -Catenin also has recently been implicated in oncogenesis. An NH₂-terminally deleted β -catenin was identified by its transformation potential in a screen for oncogenes (55). Furthermore, similar to the data from colon cancer cell lines, increased cytosolic and nuclear localization of β -catenin in adenomas and carcinomas of FAP patients has been reported (17). Our findings raise the possibility that APC may regulate cell proliferation in the colon by virtue of its role as a signal transducing protein that is associated with signaling by β -catenin. It is conceivable that the downstream targets of APC are dependent on the cell or tissue context. Thus, the same signaling activity that regulates dorsoanterior axis formation in *Xenopus* embryos could suppress or regulate growth in colon tissue. Lack of this signaling activity in the truncated mutant APC proteins would consequently result in hyperproliferation and polyp formation. Hence, the early *Xenopus* embryo might be a powerful system for elucidating mechanisms of signal transduction by APC in general.

We thank Dr. D.L. Turner in the laboratory of the late Dr. H. Weintraub for the gift of the pCS2+ expression vectors. We are grateful to Drs. F. Fagotto and R.W. Nelson for sharing their results on C-cadherin and Siamois and are indebted to Georges Wu and Dara Raspberry for their contributions in the cloning process. We also thank Alpha Yap, William Briehier, François Fagotto, and Anje Cauwels for critical reading of the manuscript, and other members of the Gumbiner laboratory for appreciated support.

This work was supported by National Institutes of Health grant GM37432 awarded to B.M. Gumbiner and by the Cancer Center Support grant NCI-P30-CA-08784. B.M. Gumbiner is a recipient of a Career Scientist Award from the Irma T. Hirsch Trust.

Received for publication 26 September 1996 and in revised form 6 November 1996.

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