Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 11109–11113, December 1993
Biochemistry

Dimer formation by an N-terminal coiled coil in the APC protein

(adenomatous polyposis coli/heptad repeat region/subdomain)

GEOFF JOSLYN*†, DIANE S. RICHARDSON‡, RAY WHITE*, AND TOM ALBER‡§¶

*Department of Human Genetics and Howard Hughes Medical Institute, University of Utah School of Medicine, Salt Lake City, UT 84132; and ‡Department of Biochemistry, University of Utah School of Medicine, Salt Lake City, UT 84132

Contributed by Ray White, August 6, 1993

ABSTRACT **Mutations in the human *APC* gene are associated with an inherited predisposition to colon cancer. *APC* codes for polypeptides of ≈2800 amino acids, with sequence homologies to coiled-coil proteins in the first 900 residues. To determine the oligomerization properties of the APC protein, we used genetic and biochemical approaches to examine the ability of APC fragments to self-associate. A subdomain comprising the first 55 amino acids of APC was found to form a stable, parallel, helical dimer, as expected for a coiled coil. The location of a key dimerization element at the N terminus of the protein supports models in which mutations in *APC* exert effects through dimerization of the mutant gene products.**

Familial adenomatous polyposis (FAP) is a dominant genetic disease defined by early onset of large numbers of colonic polyps that are precursors of colon cancer (1). FAP is caused by mutations in *APC*, a gene of unknown function located on human chromosome 5 (2–7). Alternatively spliced *APC* mRNAs encode predicted polypeptides of 2742 and 2843 amino acids, with no extensive sequence homology to other proteins. The first ≈900 residues of the APC protein, however, contain proline-free blocks with heptad repeats of hydrophobic residues characteristic of coiled coils (5, 6, 8). This pattern suggests that APC may function in association with itself or with other cellular factors.

Coiled coils consist of associated α-helices that form superhelical ropes (8). The interface between the helices generally contains hydrophobic amino acids that occur at the first and fourth positions of a recurring seven-residue motif, the heptad repeat. Polar amino acids are common at the other positions in coiled-coil sequences, with charged groups often bracketing succeeding buried hydrophobic groups. Exceptions to these patterns are commonly tolerated in long coiled coils, but short coiled coils, such as leucine zippers, can be destabilized by replacements of even single interface residues (9). Because the blocks of heptad repeat sequences in APC are relatively short and contain violations of the characteristic sequence patterns, experimental tests are needed to determine oligomerization potential.

In addition, the number and the orientation of helices in coiled coils is variable (8). Examples of parallel and antiparallel dimeric, trimeric, and tetrameric coiled coils have been reported (8, 10–12). Furthermore, some coiled coils, such as laminins (13) and the leucine zippers of Fos and Jun (14), preferentially associate with heterologous sequences. We describe here the use of genetic and biochemical methods to investigate the oligomerization potential of sequences in the first 686 residues of the APC protein. We show that the first 55 amino acids of APC are sufficient to form a stable, parallel, helical dimer, as expected for a two-stranded parallel coiled coil.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Dimerization of λ Repressor by APC Sequences. *APC* gene fragments flanked by in-frame *Sal* I and *Bam*HI restriction sites were produced by PCR using appropriate cDNA clones as templates (4). Gene fragments encoding amino acids 1–112 and 1–456 were obtained by digesting an *APC* cDNA clone in the pFLAG vector (15) with *Bam*HI and *Bst*YI or *Bam*HI and *Bgl* II, respectively. To make λ repressor fusions, the *APC* restriction fragments were inserted between the *Sal* I and *Bam*HI sites of the plasmid pJH391 (9). Automated DNA sequencing (4) confirmed the structures of the plasmids. Plasmids were introduced into *Escherichia coli* AG1688 (9), and the resulting strains were plated with 100–1000 λ phages at 37°C. The tester phage, λKH54 (9), has undergone a deletion of the cI repressor. The positive control for phage sensitivity, λ^{I434} (16), contains the phage 434 immunity region, which is not recognized by λ repressor.

Expression and Purification of APC Fragments. The DNA sequence coding for amino acids 1–55 of APC (APC-55) was amplified by PCR (4), using primers that introduced flanking restriction sites (*Nde* I at the initiator methionine and *Bam*HI after a termination codon or, alternatively, flanking *Xho* I sites). To replace Ile-55 with Cys, the change in codon 55 was included in an alternative PCR primer. The PCR products were digested with restriction enzymes and inserted into *Nde* I/*Bam*HI-digested pAED4 (17) or *Xho* I-digested pFLAG (15) expression vectors. Sequencing the inserts (5) revealed the expected order of nucleotides except for a T → A change that converted Asp-7 to Glu in the APC sequences in the otherwise wild-type FLAG fusion protein. Nonetheless, this peptide was characterized biochemically, because residue 7, which would occur on the outside of a coiled coil, was expected to have no influence on oligomerization.

The peptide APC-55 was expressed from the phage T7 promoter in the pAED4-based plasmid in *E. coli* BL21(DE3) (18). Cultures were grown at 37°C in LB medium (33) supplemented with ampicillin at 50 μg/ml. When the optical density at 600 nm reached 0.76, cultures were induced with 0.4 mM isopropyl β-D-thiogalactoside (IPTG). After 2 hr, induced cultures were harvested, resuspended in buffer containing 50 mM Tris·HCl at pH 8.0, 20% (vol/vol) glycerol, 200 mM KCl, and 0.2 mM EDTA (buffer A) and were lysed by sonication. The lysate was brought to pH 2.0 by addition of HCl and centrifuged 10 min at 10,000 rpm in a Beckman JA-20 rotor to remove cell debris and precipitated protein. The pellet was washed with buffer A, and the combined supernatants were brought to pH 8.0 with NaOH. After 2-fold dilution with water, the supernatant was loaded on a DEAE-Sephadex A-25 column and eluted with a gradient of 0.1–1.0

Abbreviations: FAP, familial adenomatous polyposis; APC, product of the *APC* (adenomatous polyposis coli) gene.

†Present address: Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104.
§Present address: Department of Molecular and Cell Biology, Stanley/Donner ASU, University of California, Berkeley, CA 94720.
¶To whom reprint requests should be addressed.

M KCl in 0.5× buffer A. The peptide was concentrated by precipitation with 70% saturated ammonium sulfate, resuspended, and dialyzed against deionized water. After dialysis, the sample was clarified by centrifugation for 10 min at 10,000 rpm in a Beckman JA-20 rotor.

The Asp-7 → Glu and Ile-55 → Cys mutant APC-55 fragments fused to the FLAG epitope (15) were named FLAG-APC-55 and FLAG-APC-CYS55, respectively. These peptides were obtained by osmotic shock of induced cultures of *E. coli* strains harboring a pFLAG derivative containing the desired *APC* sequences (15). The shock supernatants were dialyzed against 20 mM Tris·HCl, pH 7.6/150 mM NaCl (buffer B) and chromatographed on DEAE-Sephadex A-25 with a gradient of 0.15–1.0 M NaCl in buffer B.

Peptides APC-55, FLAG-APC-55, and FLAG-APC-CYS55 were further purified by HPLC (Beckman System Gold) on a C_{18} column (Vydac, 2 × 250 mm) with a linear gradient (1%/min) of 80% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid. The purity of the peptides was judged by reverse-phase chromatography on an analytical C_{18} column (Vydac, Hesperia, CA) and by electrophoresis on *N*-[tris(hydroxymethyl)methyl]glycine (Tricine)/NaDodSO$_4$ gels (19).

Absorbance and CD Spectroscopy. Peptide concentrations were determined (20) by using an Aviv 118 spectrophotometer. Extinction coefficients of peptides APC-55, FLAG-APC-55, and FLAG-APC-CYS55 were 1338, 2665, and 2725 $M^{-1}\cdot cm^{-1}$, respectively. CD spectra were recorded in buffer B, using 0.1-cm or 1.0-cm path-length cells in Aviv 62DS instruments (21). Temperature was regulated with a thermoelectric cell holder under computer control. For thermal denaturations, the temperature was changed in 2°C steps with 2-min equilibration at each temperature.

Protein Sequencing and Mass Spectrometry. The N-terminal sequences of the purified peptides were determined by using an Applied Biosystems 477A pulsed liquid protein sequencer. The results showed that the initiator methionine was absent from each peptide.

The molecular weights of the APC-55 and FLAG-APC-55 peptides were determined by electrospray ionization in a VG Fisons Trio 2000 mass spectrometer. The respective experimental m/z values of 6103.4 and 9130.25 compared favorably with the molecular weights (6103.4 for APC-55 and 9129.6 for FLAG-APC-55) calculated from the deduced amino acid sequences of the peptides.

Analytical Ultracentrifugation. APC-55 and FLAG-APC-55 peptides (in buffer B) were sedimented to equilibrium in a Beckman XL-A analytical ultracentrifuge. Radial distributions of peptides were monitored by optical absorbance at 235 or 280 nm. Partial specific volumes were calculated from the amino acid composition of each peptide, and a value of 1.008 g/cm^3 was used for the solvent density.

Exclusion Chromatography. The purified peptides were chromatographed on a Superdex 75 FPLC column (Pharmacia) in buffer B containing 1 mM EDTA. Samples were loaded at 0.5 ml/min and the column was developed at 1.7 ml/min. To ascertain the effect of disulfide bond formation on molecular weight, the FLAG-APC-CYS55 peptide was chromatographed in the presence and absence of 10 mM reduced dithiothreitol. Globular protein standards (thyroglobulin, gamma globulin, ovalbumin, and myoglobin) were obtained from Bio-Rad.

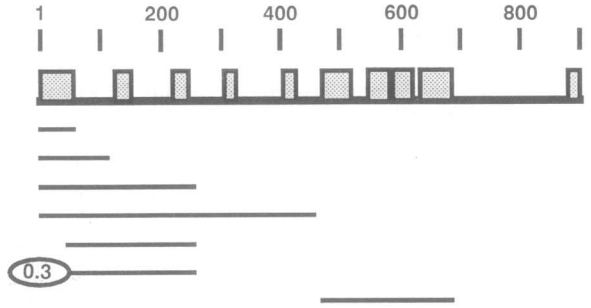
RESULTS

The dimerization potential of the APC heptad repeats was initially assessed by testing their ability to provide a dimerization domain for the cI repressor of phage λ (9). λ repressor functions as a dimer, with DNA binding and dimerization mediated by the N- and C-terminal domains, respectively. The C-terminal dimerization domain can be functionally replaced by heterologous motifs such as the GCN4 leucine zipper, a short sequence that forms a two-stranded parallel coiled coil (9, 22).

Fragments of the *APC* gene (Fig. 1) were cloned in a λ repressor test system (9), and *E. coli* strains harboring the resulting plasmids were challenged with λ KH54 (Δc*I*) and λ^{I434} phages. Resistance to λ KH54 and sensitivity to λ^{I434}, which contains operator DNA sequences not recognized by λ repressor, suggests that a functional λ-repressor-APC dimer is expressed from the test plasmid. When joined to the λ repressor DNA-binding domain, APC residues 1–55 conferred resistance to infection by λ KH54 (Fig. 1), suggesting that this first block of heptad repeats is sufficient for dimer formation.

The structure of the first block of heptad repeats was analyzed, by using APC fragments expressed in *E. coli*. As judged by mass spectrometry and N-terminal sequencing, the peptide APC-55 contained residues 2–55 of APC. The related peptide, FLAG-APC-55, contained a 24-residue, vector-derived sequence (15) N-terminal to the first 55 residues of APC (Fig. 2*A*), as well as a replacement of Asp-7 by Glu in the APC sequence. Residue 7, because of its exterior location in the predicted coiled coil (22), was expected to have no effect on oligomerization. Consequently, the FLAG-APC-55 peptide was used for structural characterizations and comparisons.

The CD spectrum of APC-55 (Fig. 2*B*) suggested that this peptide is ≈98% helical, consistent with a coiled-coil structure. At 277 μM, APC-55 showed a denaturation midpoint of



APC protein fragment	Resistance to λ phages: KH54 (Δcl)	I 434
1-55	+	-
1-112	+	-
1-256	+	-
1-456	+	-
46-256	+/-	-
Exon 0.3-46-256	+/-	-
469-686	-	-
Vector only	-	-

FIG. 1. Resistance to λ infection conferred by dimerization of APC-λ-repressor fusion proteins. The indicated APC sequences, mapped on the left, were fused C-terminal to the DNA-binding and linker domains of the λ cI protein (9), and cells containing the resulting expression plasmids were tested for their resistance to KH54 (9) and I434 (16) λ phages. The construct labeled 0.3-46-256 contains the open reading frame from the upstream exon 0.3 joined to APC residues 46–256. A + denotes resistance to phage growth, +/− indicates the growth of small plaques, and − indicates phage sensitivity. Proposed coiled-coil regions in the first 900 amino acids of the APC protein (5, 6) are shown schematically as stippled blocks.

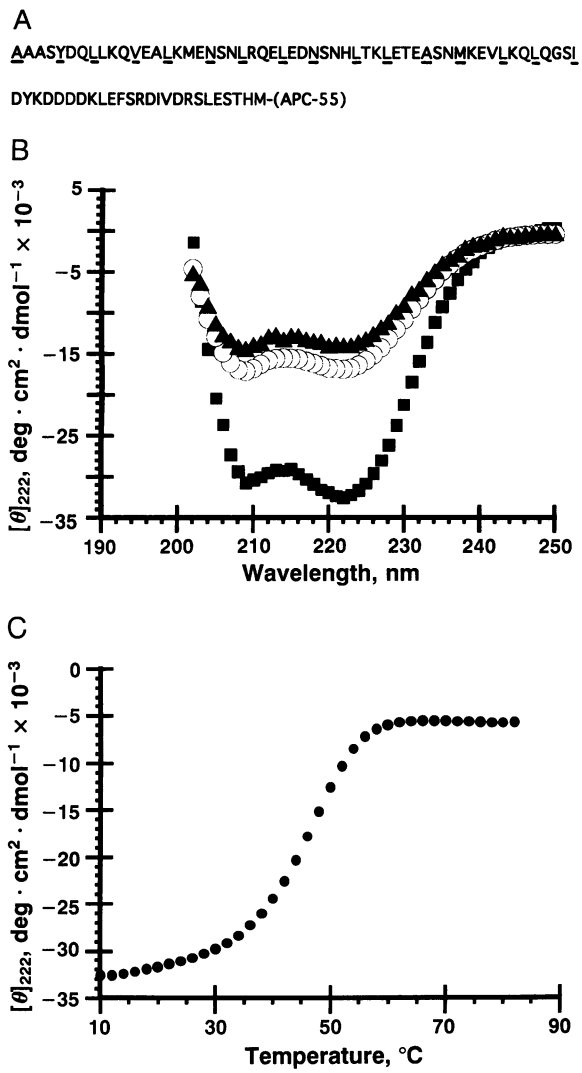


FIG. 2. (*A*) Amino acid sequences of APC-55 (upper) and FLAG-APC-55 (lower) listed in the one-letter code. FLAG-APC-55 also contained the substitution Asp-7 → Glu in the APC sequence. Residues at the characteristic hydrophobic positions of the heptad repeats are underlined. (*B*) CD spectra of APC-55 (■), FLAG-APC-55 (▲), and FLAG-APC-CYS55 (○). Peptide concentrations were 55, 19, and 73 μM, respectively, in buffer B. (*C*) Temperature dependence of the CD signal at 222 nm for APC-55. The peptide concentration was 277 μM in buffer B.

46°C at neutral pH (Fig. 2*C*). CD spectra of the fusion peptide FLAG-APC-55 (Fig. 2*B*) also are consistent with the conclusion that the APC sequence adopts a helical conformation.

To discern the oligomerization states of APC-55 and FLAG-APC-55, we compared their molecular weights in solution to the molecular weights of the peptide monomers. For both peptides, the molecular weights in solution, measured by equilibrium analytical ultracentrifugation, were approximately twice the monomer molecular weights determined by mass spectrometry and peptide sequencing (Table 1). These results confirmed the neutrality of the Asp → Glu substitution in FLAG-APC-55 and indicated that the N-terminal peptides of APC form homodimers.

The orientation of the helices in the APC dimer was determined by using a mutant fusion peptide (FLAG-APC-CYS55) with a single cysteine introduced in place of Ile-55. In a parallel coiled coil, cysteine at this position can form a disulfide bond in the dimer (23). In an antiparallel coil, however, disulfide-bonded peptides are expected to form tetramers or larger aggregates (Fig. 3*A*). The FLAG-APC-CYS55 mutant readily formed a disulfide bond in aqueous solution. This was apparent from the absence of a color change upon titration of the purified peptide with dithionitrobenzoic acid (25) and by the quantitative conversion of the peptide to a covalent dimer in the absence of reducing agent (Fig. 3*B*). The disulfide-bonded mutant peptide and the cysteine-free FLAG-APC-55 gave similar CD spectra (Fig. 2*B*), indicating that the secondary structure is unaffected by the disulfide. Importantly, the disulfide bond in FLAG-APC-CYS55 did not change the size of the peptide oligomer determined by exclusion chromatography (Fig. 3*C*), suggesting that the disulfide bond linked monomers within the dimer. Thus, the orientation of the helices is parallel.

Table 1. Oligomerization of APC peptides

Peptide	Conc., μM	M_r in solution	Ratio of solution M_r to monomer M_r
APC-55	35	11,100	1.82
	100	12,200	2.00
	200	11,500	1.88
FLAG-APC-55	7.7	17,900	1.96
	77	19,200	2.11

Molecular weights (M_r) in solution, determined by analytical ultracentrifugation, were compared with the monomer molecular weight (6103.2 for APC-55 and 9129.6 for FLAG-APC-55) calculated from the amino acid sequences of the peptides.

DISCUSSION

The studies reported here show that the first block of heptad repeats in the APC protein is capable of forming a stable, parallel, helical dimer. These properties, together with the observation that residues 1–55 show the best coiled-coil homology in the sequence (6, 8), strongly favor a two-stranded coiled-coil structure for the N terminus of the APC protein.

Although our results have not fully defined the extent of the dimerization domain, the N-terminal coiled coil is likely to be a key dimerization element. For example, fusion to residues 469–686 of APC, which contain four blocks of proposed heptad repeats (4, 6), did not activate the λ repressor DNA-binding domain (Fig. 1). In addition, efficient dimerization depends on amino acids 1–45, encoded by exon 1 of *APC*. Motivated by the discovery of additional 5′ *APC* exons and alternatively spliced mRNAs lacking exon 1 (A. Thliveris, W. Samowitz, and R.W., unpublished data), we constructed two λ-repressor-APC fusions lacking exon 1 sequences (Fig. 1). The first fusion contained APC amino acids 46–256. The second, which reflects alternative splicing of the APC message, has the λ repressor DNA-binding domain joined to the open reading frame from the 5′ exon 0.3 joined directly to exon 2 and ends at codon 256. *E. coli* harboring either of these constructs exhibited small plaques when challenged with λ KH54 (Fig. 1). In contrast, the λ repressor fusion to APC residues 1–256 rendered cells immune to infection. The reduced activity of λ repressor fusions lacking residues encoded by exon 1 is difficult to interpret in the absence of assays for expression and folding of the chimeric proteins. Nonetheless, the results underscore the importance of residues 1–45 for dimerization of the APC N terminus and are consistent with the suggestion that oligomerization of APC may be regulated by alternative splicing.

These results complement recent immunochemical studies showing that wild-type and C-terminally truncated APC proteins associate *in vivo* and *in vitro* (26, 27). Western blot experiments, for example, revealed full length and truncated APC proteins larger than M_r 80,000 in normal and cancerous tissues and cell lines. In addition, APC polypeptides as short as the first 171 amino acids formed a complex with the N-terminal 1013 residues after the fragments were expressed

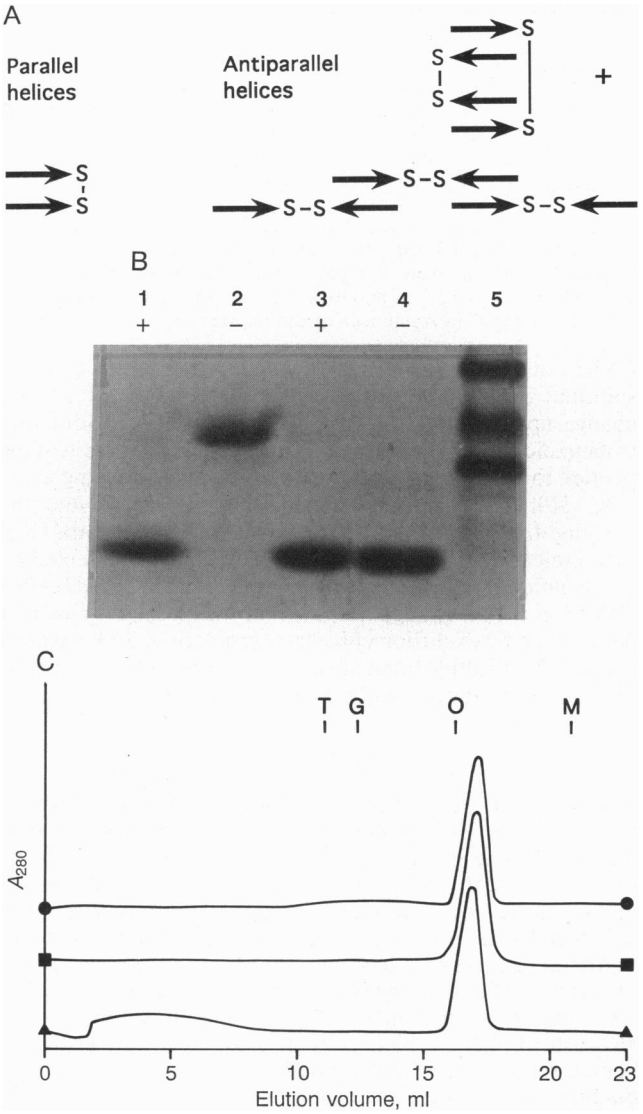


FIG. 3. (*A*) Oligomerization of disulfide-bonded peptides in parallel and antiparallel coiled coils. Parallel helices can form disulfide-bonded dimers, but antiparallel helices must associate in higher-order disulfide-bonded oligomers. (*B*) Denaturing gel of FLAG-APC peptides. Lanes 1 and 2, FLAG-APC-CYS55 with (+) and without (−) 100 mM reduced dithiothreitol in the sample buffer. Lanes 3 and 4, FLAG-APC-55 (wild-type) with (+) and without (−) 100 mM reduced dithiothreitol in the sample buffer. Lane 5, prestained standards (hen lysozyme, soybean trypsin inhibitor, and carbonic anhydrase) with mobilities corresponding to molecular weights of 18,500, 27,500, and 32,500, respectively. (*C*) Exclusion chromatography of peptides on a Superdex 75 FPLC column, in buffer B containing 1 mM EDTA. FLAG-APC-CYS55 was chromatographed in the absence (■) and presence (●) of 10 mM reduced dithiothreitol. The control peptide, FLAG-APC-55 (▲), was shown to be dimeric by analytical ultracentrifugation (Table 1). Elution volumes of four globular proteins, thyroglobulin (T; M_r = 670,000), gamma globulin (G; M_r = 158,000), ovalbumin (O; M_r = 44,000), and myoglobin (M; M_r = 17,000), are indicated. Because of their elongated shapes, dimeric coiled-coil peptides migrate faster than globular proteins of equal molecular weight (24). Formation of a disulfide bond between Cys-55 residues does not alter the mobility of the peptide.

together *in vitro* (27). This association, like the dimerization activity of APC fragments in the λ repressor fusions (Fig. 1), depended on the first 45 residues of the protein.

Deletion of the first 45 amino acids encoded by exon 1 leaves only 10 residues of the first block of heptad repeats. Because such a short coiled-coil sequence is expected to be unfolded (24), the residual activity of the λ repressor fusion to residues 46–256 of APC (Fig. 1), together with the modest thermal stability of the APC-55 peptide, could reflect dimer contacts beyond residue 55. We attempted to test this possibility by measuring the thermal stability of a purified peptide containing APC residues 1–205. Direct comparisons to the denaturation of APC-55 were impossible, however, because the thermal denaturation of the 1–205 polypeptide was partially irreversible under the conditions tested (data not shown).

It is striking that the coiled-coil dimerization motif occurs at the N terminus of APC. More than 90% of the disease alleles of *APC* characterized to date contain base changes or frameshifts that introduce premature termination codons (5, 7, 28, 29). All of these mutations have occurred 3′ of the region coding for the N-terminal coiled coil characterized here. Because the resulting truncated proteins retain the N-terminal dimerization domain, it is possible, as has been suggested (26, 27, 30, 31), that the association of truncated with full-length gene products plays a role in the effect of the *APC* mutations. Alternatively, the truncated APC polypeptides might form partially active homodimers capable of inhibiting the functions of wild-type dimers.

The idea that truncated polypeptides play a role in the etiology of FAP is supported by the finding that different truncations are associated with different disease symptoms. For example, the number of colonic polyps is usually much reduced in individuals who carry the most 5′ mutations yet discovered, frameshifts at amino acids 73 and 77 (ref. 32; L. Spirio and R.W., unpublished data). If the truncated APC peptides self-associate or associate with the wild-type protein in heterozygotes, the attenuated phenotype would suggest that sequences C-terminal to residue 77 may promote more severe disease symptoms by mediating more deleterious interactions with the normal APC protein or with products of other genes.

Finally, our finding that formation of homodimers requires only the first 55 amino acids of APC suggests that the coiled-coil peptides discussed here might be used in lieu of specific antibodies to assay directly for APC polypeptides. Because stable truncated APC proteins have been detected in FAP patients and colon tumor cell lines (26, 27), peptides that associate with the N-terminal dimerization domain may serve as effective diagnostic tools.

We thank James Hu for providing the λ repressor dimerization assay system; Naomi Franklin for λ^{I434}; Lino Gonzalez, Ying Yang, and Howard Schachman for performing the sedimentation analyses; Margaret Robertson for DNA sequencing; Ed Meenan for DNA synthesis; Peter Kim and Tao Zhang for help with CD measurements; and James Hu, Peter Kim, and Lisa Spirio for helpful discussions. We appreciate the careful review of the manuscript by Jean Marc Lalouel and Ray Gesteland. We are grateful to Robert Schackmann of the Protein/DNA Core Facility of the Utah Cancer Center for peptide sequencing and HPLC. We also thank Pam Crain for recording and analyzing mass spectra at the University of Utah Mass Spectrometry Facility. This work was supported by grants from the American Cancer Society (NP-815C) to T.A. and from the National Institutes of Health (RO1-HG00367-04) to R.W.; R.W. is an Investigator of the Howard Hughes Medical Institute.

1. Burt, R. & Samowitz, W. (1988) *Gastroenterol. Clin. North. Am.* **17**, 657–678.
2. Bodmer, W., Bailey, C., Bodmer, J., Bussey, H., Ellis, A., Gorman, P., Lucibello, F., Murday, V., Rider, S., Scambler, P., Sheer, D., Solomon, E. & Spurr, N. (1987) *Nature (London)* **328**, 614–616.
3. Leppert, M., Dobbs, M., Scambler, P., O'Connell, P., Nakamura, Y., Stauffer, D., Woodward, W., Burt, R., Hughes, J., Gardner, E., Lathrop, M., Wasmuth, J., Lalouel, J.-M. & White, R. (1987) *Science* **238**, 1411–1413.
4. Joslyn, G., Carlson, M., Thliveris, A., Albertsen, H., Gelbert,

L., Samowitz, W., Groden, J., Stevens, J., Spirio, L., Robertson, M., Sargeant, L., Krapcho, K., Wolff, E., Burt, R., Hughes, J., Warrington, J., McPherson, J., Wasmuth, J., Le Pasilier, D., Abderrahim, H., Cohen, D., Leppert, M. & White, R. (1991) *Cell* **66**, 601–613.
5. Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M., Sargeant, L., Krapcho, K., Wolff, E., Burt, R., Hughes, J., Warrington, J., McPherson, J., Wasmuth, J., Le Paslier, D., Abderrahim, J., Cohen, D., Leppert, M. & White, R. (1991) *Cell* **66**, 589–600.
6. Kinzler, K., Nilbert, M., Su, L.-K., Vogelstein, B., Bryan, T., Levy, D., Smith, K., Preisinger, A., Hedge, P., McKechnie, D., Finnear, R., Markham, A., Groffen, J., Boguski, M., Altschul, S., Horii, A., Ando, H., Miyoshi, Y., Miki, Y., Nishisho, I. & Nakamura, Y. (1991) *Science* **253**, 661–665.
7. Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K., Utsunomiya, J., Baba, S., Hedge, P., Markham, A., Krush, A., Petersen, G., Hamilton, S., Nilbert, M., Levy, D., Bryan, T., Preisinger, A., Smith, K., Su, L.-K., Kinzler, K. & Vogelstein, B. (1991) *Science* **253**, 665–669.
8. Cohen, C. & Parry, D. (1986) *Trends Biochem. Sci.* **11**, 245–248.
9. Hu, J. C., O'Shea, E. K., Kim, P. S. & Sauer, R. T. (1990) *Science* **250**, 1400–1403.
10. Banner, D. W., Kokkinidis, M. & Tsernoglou, D. (1987) *J. Mol. Biol.* **196**, 657–675.
11. Cusack, S., Berthet-Colominas, C., Hartlein, M., Nassar, N. & Leberman, R. (1990) *Nature (London)* **347**, 249–255.
12. Lovejoy, B., Choe, S., Cascio, D., McRorie, D. K., DeGrado, W. F. & Eisenberg, D. (1993) *Science* **259**, 1288–1293.
13. Hunter, I., Schulthess, T. & Engel, J. (1992) *J. Biol. Chem.* **267**, 6006–6011.
14. O'Shea, E. K., Rutkowski, R., Stafford, W. F., III, & Kim, P. S. (1989) *Science* **245**, 646–648.
15. Hopp, T. P., Prickett, K. S., Price, V. L., Libby, R. T., March, C. J., Cerretti, D. P., Urdal, D. L. & Conlon, P. J. (1988) *Bio/Technology* **6**, 1204–1210.
16. Kaiser, A. D. & Jacob, F. (1957) *Virology* **4**, 509–521.
17. Doering, D. S. (1992) Dissertation (Massachusetts Institute of Technology, Cambridge).
18. Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorf, J. W. (1990) *Methods Enzymol.* **185**, 60–89.
19. Schaegger, H. & von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379.
20. Gill, S. C. & von Hippel, P. H. (1989) *Anal. Biochem.* **182**, 319–326.
21. O'Shea, E. K., Rutkowski, R. & Kim, P. S. (1989) *Science* **243**, 538–542.
22. O'Shea, E. K., Klemm, J. D., Kim, P. S. & Alber, T. (1991) *Science* **254**, 539–544.
23. Zhou, N. E., Kay, C. M. & Hodges, R. S. (1992) *Biochemistry* **31**, 5739–5746.
24. Lau, S. Y. M., Taneja, A. K. & Hodges, R. S. (1984) *J. Biol. Chem.* **259**, 13253–13261.
25. Creighton, T. E. (1990) in *Protein Structure: A Practical Approach*, ed. Creighton, T. E. (Oxford Univ. Press, Oxford), pp. 155–167.
26. Smith, K. J., Johnson, K. A., Bryan, T. M., Hill, D. E., Markowitz, S., Willson, J. K. V., Paraskeva, C., Petersen, G. M., Hamilton, S. R., Vogelstein, B. & Kinzler, K. W. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 2846–2850.
27. Su, L.-K., Johnson, K. A., Smith, K. J., Hill, D. E., Vogelstein, B. & Kinzler, K. W. (1993) *Cancer Res.* **53**, 2728–2731.
28. Groden, J., Gelbert, L., Thliveris, A., Nelson, L., Robertson, M., Joslyn, G., Samowitz, W., Spirio, L., Carlson, M., Burt, R., Leppert, M. & White, R. (1993) *Am. J. Hum. Genet.* **52**, 263–272.
29. Miyoshi, Y., Ando, H., Nagase, H., Nishisho, I., Horii, A., Miki, Y., Mori, T., Utsunomiya, J., Baba, S., Petersen, G., Hamilton, S., Kinzler, K., Vogelstein, B. & Nakamura, Y. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 4452–4456.
30. Bourne, H. (1991) *Nature (London)* **351**, 188–190.
31. Bourne, H. (1991) *Nature (London)* **353**, 696–697.
32. Spirio, L., Otterud, B., Stauffer, D., Lynch, H., Lynch, P., Watson, P., Lanspa, S., Smyrk, T., Cavalieri, J., Howard, L., Burt, R., White, R. & Leppert, M. (1992) *Am. J. Hum. Genet.* **51**, 92–100.
33. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), p. 440.