Sequence-based design of a peptide probe for the APC tumor suppressor protein

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Background: Proteins form specific associations, but predictive rules for protein pairing are generally unknown. Here, we describe amino-acid sequence patterns capable of mediating specific pairing of a widespread protein motif: the parallel, dimeric, α-helical coiled coil. The pairing rules were tested by designing a 54-residue peptide (anti-APCp1) that is predicted to dimerize preferentially with a coiled-coil sequence from the adenomatous polyposis coli (APC) tumor suppressor protein.

Results: As judged by circular dichroism, ultracentrifugation and native gel electrophoresis, anti-APCp1 formed a specific, helical, dimeric complex with the target APC coiled coil. On western blots of APC fragments expressed in *Escherichia coli*, the designed peptide detected a pattern of bands identical to the pattern detected by an antibody directed against the APC coiled coil. Peptide-mediated precipitation experiments showed that anti-APCp1 bound and sequestered wild-type and mutant APC proteins in extracts of human colon cancer cell lines. In addition, binding of the designed peptide preserved native APC–β-catenin complexes.

Conclusions: These biochemical experiments demonstrate that the anti-APC peptide preferentially forms a heterodimeric coiled coil with mutant and full-length APC proteins. The specificity of the designed peptide is sufficient to support several applications that commonly use antibodies. The observed specificity of anti-APCp1 validates the pairing rules used as the basis for the probe design, and it suggests that residues in the core positions of coiled coils help impart pairing selectivity.

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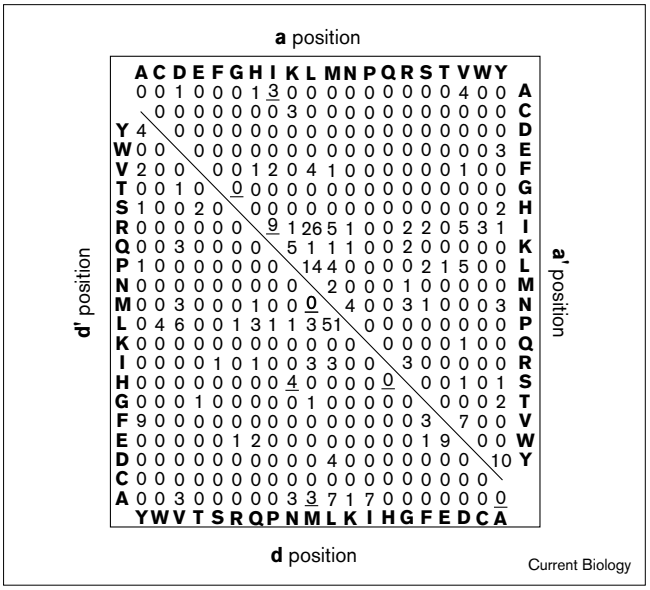
Background

Specific molecular probes, such as antibodies and oligonucleotides, have broad applications in molecular biology and medicine. The advent of new probes targeted for proteins, however, is limited by the absence of generalizable recognition rules. Oligomerization specificity has been especially well studied for the coiled coil, a simple structural motif in which α-helices wrap around each other in a left-handed supercoil [1–4]. Approximately 1–2% of the amino acids in proteins occur in coiled coils [1,3], which are found in diverse functional contexts including transcription factors, enzymes, motors, muscle proteins, intermediate filaments, cellular and viral surface proteins and tumor suppressors [1–3]. Coiled-coil sequences are characterized by a seven-residue repeat, $(\mathbf{a}\,\mathbf{b}\,\mathbf{c}\,\mathbf{d}\,\mathbf{e}\,\mathbf{f}\,\mathbf{g})_n$, in which the first (**a**) and fourth (**d**) positions comprise the hydrophobic core of the interhelical interface and, generally, polar residues at **e** and **g** flank the core. Although they share a common structure, dimeric coiled coils pair specifically [5]. This specificity has been demonstrated in wild-type protein sequences [6–8] and synthetic model systems [9–11].

To develop pairing rules that could guide the design of complementary coiled-coil peptides, we relied on thermodynamic measurements of specificity [5,12–14] and covariation patterns in coiled-coil sequences (Figure 1). In both natural and model coiled coils, specific associations have been attributed to destabilizing ionic interactions in the homodimers that are replaced with more favorable contacts in the heterodimer [5,12–18]. Consequently, our peptide design included charged amino acids at **g** and succeeding **e′** positions that could form ion pairs in the heterodimer and repulsive ionic interactions in the designed peptide homodimer [12].

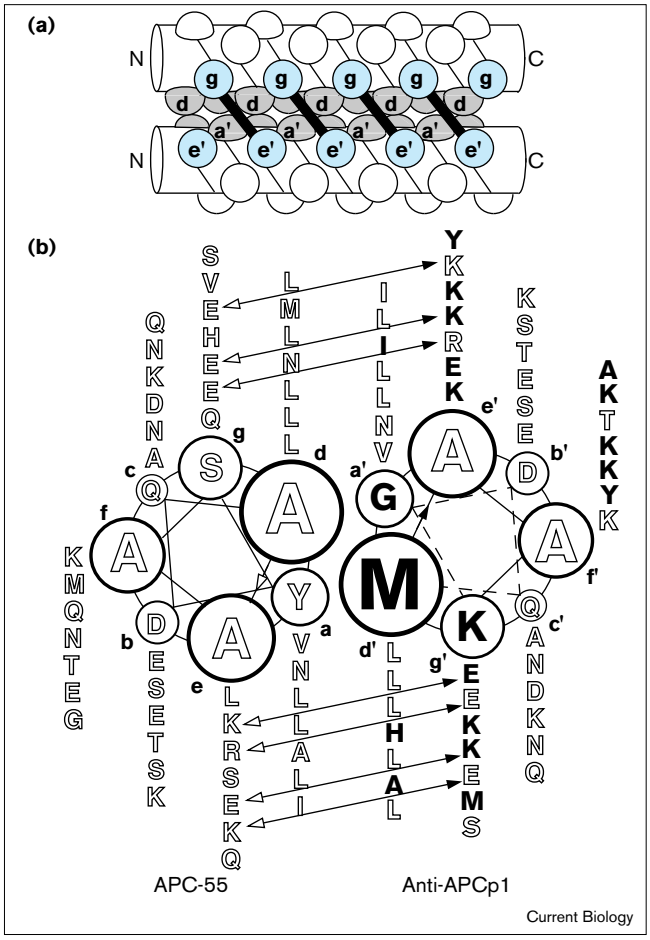
In contrast to the **g** and **e** positions, little specificity has been ascribed to core residue contacts [5,7,19,20]. In the heterodimer formed by the Fos and Jun leucine zippers, for example, three heterotypic pairs of core residues appear to impart none of the observed preference of the chains for each other [5]. This is surprising considering that the core residues form the majority of interhelical contacts [4] and that mutations of core residues have dramatic effects on stability and oligomerization [21–26]. To

Figure 1



Covariation in the core residues of coiled coils in the heterodimeric cytokeratins. Core amino acids are listed in the single-letter code, and the number of occurrences of each interhelical **a**–**a′** (top) and **d**–**d′** (bottom) residue pair in the heterodimers is tabulated. Numbers on the diagonals denote homotypic amino-acid pairs in the heterodimer sequences. Amino acids with a high relative frequency of heterotypic pairing and a low relative frequency of homotypic pairing – such as Ala, Ile, Met and Ser at the **a** position and Ala, Phe, His, Ile, Met, Asn and Val at the **d** position – provide evidence that core residues can confer specificity to coiled-coil dimerization. Off-diagonal combinations expected to favor the heterodimer and homotypic pairs expected to disfavor the designed homodimer are underlined.

Figure 2



Contacts that form the basis for the anti-APCp1 design. **(a)** Schematic diagram of a coiled coil showing alternating **a**–**a′** and **d**–**d′** layers (gray) in the core and surrounding **g** and succeeding **e′** residues (blue) that can form interhelical electrostatic interactions (black bars). White balls correspond to surface **b**, **c** and **f** positions that do not make substantial interhelical contacts. Supercoiling of the two helices is omitted for simplicity. **(b)** Helical wheel diagram showing the sequences of the target APC-55 peptide (left) and the designed anti-APCp1 probe (right). The residues in the wild-type APC-55 peptide are shown in outlined letters, and the sequence changes in anti-APCp1 are shown in bold. The sequences begin with Ala2 (APC-55) and Met2 (anti-APCp1) at position **d**. The view is from the amino terminus along the superhelical axis. The **a**–**a′** and **d**–**d′** pairs form alternating layers, and potential interhelical ion pairs between **g** and succeeding **e′** residues are marked with arrows.

obtain principles for core complementation, we analyzed the sequences of the coiled-coil domains in cytokeratins that form obligate heterodimers [27,28]. Uneven occurrences of heterotypic and homotypic pairing provided evidence for core-mediated specificity and revealed the identities of preferred interhelical neighbors (Figure 1).

The ability of these two simple patterns — complementary (and repulsive) charges and heterotypic core contacts — to direct heterotypic pairing was investigated by designing a peptide complementary to the coiled-coil domain of the adenomatous polyposis coli (APC) tumor suppressor protein. Mutations in the APC gene are associated with 75% of sporadic colon tumors [29] and with a familial predisposition to colon cancer [30]. Alternative mRNA splicing predicts a family of large APC proteins (~2800 amino acids) that are thought to regulate cell adhesion and transcription in part by binding and down-regulating β-catenin [31–35]. The amino-terminal 55 amino acids of the APC protein form a coiled coil that mediates homodimerization [36,37]. The majority of disease-associated mutations predict truncated proteins [29,30,38]; therefore, tumorigenic as well as wild-type APC proteins contain the coiled-coil domain targeted by our anti-APC peptide.

Results and discussion

To design the anti-APC peptide (anti-APCp1), we made 20 mutations in the sequence of the first 55 amino acids of APC (Figure 2). Five changes were made in the **a** and **d** positions — Ala2Met, Tyr6Gly, Asn30His, Ala41Ile and Met44Ala (Figure 2b) — based on covariation patterns at **a** and **d** positions of keratin type I and type II heterodimers

(Figure 1). Eight changes at **g** and **e** positions — Ser5Lys, Leu10Lys, Gln12Glu, Lys17Glu, Glu26Lys, Ser31Lys, His33Lys, and Glu38Lys (Figure 2b) — were made to destabilize anti-APCp1 homodimers and form ion pairs upon heterodimerization with APC. Two additional changes at the **e** and **g** positions — Val47Met and Gln52Tyr — were made to increase stability [39] and introduce a chromophore.

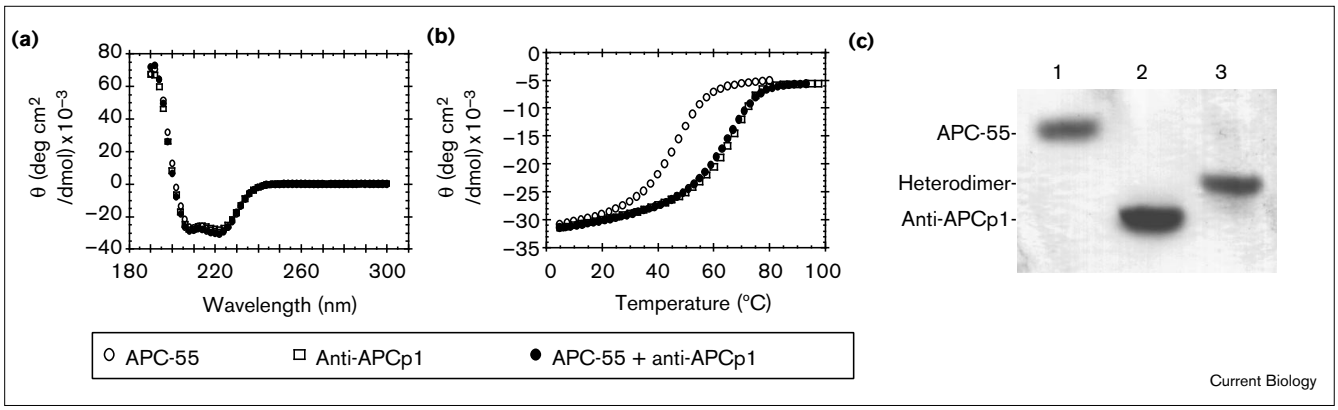
Because they make no interhelical contacts, changes at the **f** positions have little effect on pairing specificity [4]. Gln25, Asn32, and Glu46 at **f** positions were changed to Lys to increase net charge to aid in separation of the heterodimer. These mutations also increased the helical propensity of the complementary sequence [39]. The **f** position changes Gly53Ala and Met18Tyr were made to reduce terminal fraying [40] and add a chromophore, respectively.

The preferential interaction of anti-APCp1 with the APC amino terminus was demonstrated *in vitro* by thermal denaturation, sedimentation equilibrium and native gel electrophoresis. Circular dichroism (CD) spectra indicated that the wild-type APC amino terminus (APC-55), the designed peptide probe and an equimolar mixture of the two peptides were at least 95% helical (Figure 3a). The apparent melting temperature (T_m) of the heterodimer was more than 10°C higher than the average of the two homotypic species at half the concentration, suggesting that the anti-APCp1 peptide preferentially formed heterodimers with the wild-type APC-55 (Table 1). The increased thermal stability of the heteromeric mixture was observed at different peptide concentrations (Table 1), consistent with formation of hetero-oligomers.

Equilibrium sedimentation studies confirmed the heteromeric mixture to be a dimer, as expected from our dimeric coiled-coil design (Table 1). Anti-APCp1 by itself, however, formed a mixture of oligomers in solution, as indicated by an average molecular weight larger than expected for a dimer (Table 1) and systematic residuals from a dimer fit (data not shown). Consequently, the dimeric nature of the 1:1 mixture of APC-55 and anti-APCp1 suggested that binding to the wild-type target peptide reduced the concentration of higher order oligomers of the probe.

The most direct measure of heterodimer specificity was provided by native acrylamide gel electrophoresis. Compared with the APC-55 and anti-APCp1 peptides alone, an equimolar mixture of the two peptides yielded a distinct species with intermediate mobility (Figure 3c). The appearance of the heterodimer band with concomitant loss of both homomeric species demonstrates the high specificity of the designed peptide. Urea denaturation at 20°C and pH 7.0, monitored by CD, suggested upper limits for the dissociation constants (Kds) of APC-55, anti-APCp1 and the APC-55–anti-APCp1 heterodimer of 6 nM, 0.4 nM and 0.3 nM, respectively (data not shown). Thus, the affinity of anti-APCp1 for APC-55 is 20-fold greater than the wild-type affinity.

Figure 3



The APC-55–anti-APCp1 complex is helical, thermally stable and specific. **(a)** CD spectra at 75 μM total peptide concentration of APC-55, anti-APCp1 or an equimolar mixture of APC-55 and anti-APCp1. All three species are greater than 95% helical, consistent with the coiled-coil design. **(b)** Thermal denaturation monitored by CD at 222 nm of APC-55 and anti-APCp1 peptides and an equimolar mixture of the peptides. The mixture of the two peptides shows an increase in T_m of 8°C over the wild-type homodimer. **(c)** Native gel electrophoresis: lane 1, APC-55; lane 2, anti-APCp1; lane 3, an equimolar mixture of APC-55 and anti-APCp1. The mixture of the two peptides yields a new species with intermediate mobility, and neither of the two homomeric species is detected. The mobilities of the individual peptides and the equimolar mixture are consistent with calculated isoelectric points of 10.8 (anti-APCp1), 10.0 (heterodimer) and 4.8 (APC-55). The single band for the anti-APCp1 peptide may reflect a common electrophoretic mobility for dimer and trimer species or a shift in oligomerization equilibrium at the pH (4.0) of the gel. The detection limit of the Coomassie stain gives a lower limit of ~100:1 for the specificity of anti-APCp1 for APC-55.

Table 1

Peptide oligomerization and stability.

Peptide	Apparent Mr (Da)*	Apparent T_m (± 1°C) 8.0 μM	20.0 μM	75.0 μM
APC-55	12,400	36.3	40.0	46.5
Anti-APCp1	14,000	51.5	58.6	66.4
APC-55–anti-APCp1	13,300	52.9	57.5	63.6

*Molecular weights were analyzed by equilibrium centrifugation. The expected molecular weights of the APC-55 homodimer, anti-APCp1 homodimer, and the APC-55–anti-APCp1 heterodimer were 12,000, 12,500 and 12,300 Da, respectively. Apparent molecular weights (Mr) for three different concentrations of each species were calculated and averaged. The lack of systematic residuals to a dimer fit (data not shown) indicates that both the wild-type peptide and the APC-55–anti-APCp1 mixture form dimers. In contrast, the anti-APCp1 peptide alone forms a mixture of oligomers. Apparent melting temperature (T_m) was determined by CD at 222 nm. The observed increase in apparent T_m for all species with increased concentration is consistent with the formation of oligomers.

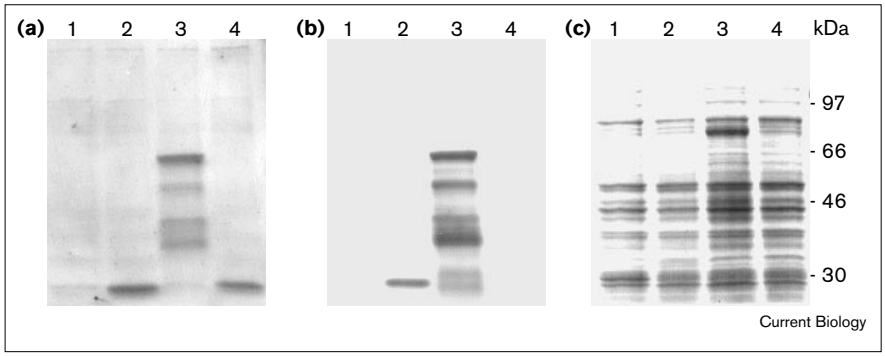
The ability of anti-APCp1 to distinguish APC from other cellular proteins and compete effectively with homotypic pairs of full-length APC subunits was investigated by western blotting and affinity purification. Anti-APCp1 and antibodies against the amino terminus of APC detected similar patterns of glutathione-S-transferase (GST)–APC fusion proteins expressed in *Escherichia coli* (Figure 4). Western blots developed with either the designed peptide or the antibody revealed common regions of proteolytic susceptibility within the first 373 amino acids of APC (Figure 4, lane 3). In contrast to the antibody, the anti-APCp1 peptide also bound to a GST fusion protein containing the designed probe sequence itself (Figure 4, lane 4). Thus, the 20 sequence changes in anti-APCp1 apparently abolished the antibody epitope but preserved the capacity for homo-oligomerization. These results suggest not only that the designed peptide forms a coiled-coil interaction with the APC amino terminus, but also that this coiled coil forms in preference to interactions with *E. coli* proteins.

Because of its large size (approximately 312 kDa) and low abundance [41], detection of APC protein in human cells presents special challenges. No APC species were detected when peptide-mediated or antibody-mediated precipitates of human tumor cell lines were separated by gel electrophoresis and silver-stained or blotted and stained with Ponceau S (data not shown). Moreover, autoradiographs of precipitations from [^{35}S]methionine-labeled lysates revealed a similar pattern of bands when either biotinylated anti-APCp1 or biotin alone were used for precipitation (data not shown).

Low endogenous amounts of full-length and mutant APC polypeptides were detected, however, by western blotting peptide-mediated precipitations from colon cancer cell lines (Figure 5). Similar patterns and yields of APC polypeptides were captured with biotinylated anti-APCp1 or a biotinylated monoclonal antibody specific for the APC amino terminus (Figure 5a). No APC was detected when affinity precipitations were carried out with the peptide alone (Figure 5, lane 3) or biotin alone (Figure 5, lane 4). Thus, the affinity precipitation of APC was specific to the biotin–anti-APCp1 conjugate. Because the amino-terminal, coiled-coil sequence targeted by anti-APCp1 is present in tumor-associated truncation mutants, the distinct electrophoretic mobilities of the species detected by the designed peptide distinguish wild-type from tumorigenic APC variants (Figure 5).

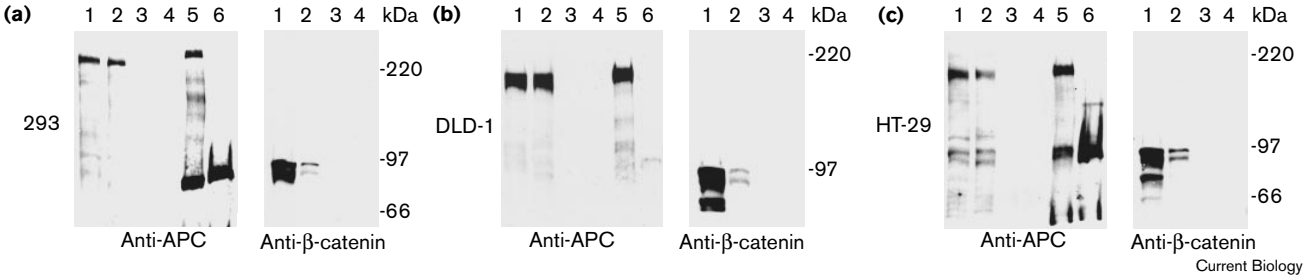
These results indicate that the biotinylated anti-APCp1 peptide pairs effectively with full-length APC polypeptide chains. As previously reported for APC-specific antibodies [31,33], the anti-APCp1 peptide coprecipitated

Figure 4



Western blots of APC fusion proteins expressed in *E. coli*. Blots were probed using either **(a)** biotinylated anti-APCp1, or **(b)** a monoclonal antibody, AB-1, that recognizes amino acids 1–29 of the APC protein. **(c)** The Coomassie-stained gel used to generate the blots. The molecular weights of protein standards are indicated in kDa. (a–c) Lane 1, GST only; lane 2, GST–APC-55; lane 3, GST–APC-373 (GST fusion with the first 373 amino acids of APC); lane 4, GST–anti-APCp1. The biotinylated anti-APCp1 detects the same APC fragments as the antibody – lanes 2 and 3 in (a,b) – and also binds GST–anti-APCp1 – lane 4 in (a). The designed peptide distinguishes APC sequences from all other *E. coli* proteins on the blot.

Figure 5



Western blots of precipitations of APC from human colon cancer cell lines. APC was precipitated from three different cell lines: **(a)** 293, which expresses full-length APC, **(b)** DLD-1, which contains a stop at codon 1417 and expresses a truncated APC molecule of 190 kDa, and **(c)** HT-29, which expresses two truncated APC molecules of approximately 100 and 200 kDa [53]. Blots were probed with an APC antibody or a β-catenin antibody as shown. The total lysate is shown in lane 1. Precipitations were performed using: lane 2, biotinylated anti-APCp1; lane 3, anti-APCp1 without biotin; lane 4, biotin alone; lane 5, the anti-APC antibody Ab-1; and lane 6, a non-specific mouse immunoglobulin G. The molecular weights of protein standards are indicated in kDa. The biotinylated anti-APCp1 peptide precipitated truncation mutants and full-length APC at endogenous cell concentrations, demonstrating the affinity and specificity of the designed probe. The biotinylated peptide also coprecipitated two β-catenin species (right panels) previously demonstrated to associate with APC [31–35].

β-catenin (Figure 5), a protein that recognizes motifs in the central third of the APC sequence [31,33]. Although binding of anti-APCp1 to endogenous APC protein disrupts the homodimeric, amino-terminal APC coiled coil (Figure 3), APC–β-catenin complexes survived this disruption (Figure 5). Moreover, only a subset of β-catenin species coprecipitated from tumor cell lines (Figure 5), suggesting that anti-APCp1 does not bind directly to every protein in the extracts. Consistent with this conclusion, antibodies specific for E-cadherin or the 20S proteasome p32 subunit failed to stain proteins precipitated by anti-APCp1 (data not shown).

Conclusions

The specificity and stability of the anti-APCp1 heterodimer validate the two key design principles of optimized electrostatic interactions and complementary core packing. The interpretation of core contact patterns (Figure 1) is hampered by the modest number of heptad pairs, the homologies among the underlying keratin sequences and the assumption that the sequence context exerts a limited influence on the pair preference. Moreover, although the covariation analysis reflects only **a**–**a′** and **d**–**d′** pairs, some core sidechains (for example, charged residues) are accommodated in coiled coils by interactions with residues in other heptad positions [7,42]. Coiled-coil stability and oligomerization state are sensitive to core mutations [5,23,43]. In this context, the stability of the APC-55–anti-APCp1 heterodimer supports the efficacy of our design method. Greater understanding of the basis for pairing specificity will undoubtedly lead to better design rules.

In contrast to anti-APCp1, other protein-targeted ligands require experimental selections. Iterative, system-specific binding assays are necessary to obtain protein ligands from phage display or chemical libraries, and antibodies undergo selection *in vivo* [44,45]. Unlike antibodies, complementary coiled-coil peptides are small, relatively inexpensive, and stable without disulfide bonds. The designed coiled coils also necessarily disrupt the target structure [46–50]. Heterotypic coiled-coil peptides combine the utility of a sequence-based design and the simplicity of chemical synthesis. These properties may facilitate diverse applications *in vitro* and *in vivo*. Coiled-coil sequences are readily identified by computer methods [1–3], making this large family of proteins amenable to complementary peptide design.

Materials and methods

Sequence covariation analysis and complementary peptide design

Heptad repeats in the sequences of obligate heterodimers of type I and type II keratins [27,28] were assigned by the program PAIRCOIL [3] and 312 heptads were aligned by homology. Four pairs (comprising four known interacting heterodimers) of human keratin sequences were used in the analysis: K8–K18, K4–K13, K5–K14 and K1–K10. Sequences are available in the Swissprot database with the following accession numbers: K8 (P05787), K18 (P05783), K4 (P19013), K13 (P13646), K5 (P13647), K14 (P02533), K1 (P04264), and K10 (P13645). Of the twenty changes in anti-APCp1, five were made to the **a** and **d** positions based on covariation sequence analysis and eight changes at the **e** and **g** positions were made to introduce repulsive ionic interactions in the anti-APC homodimer that are relieved in the parallel heterodimer. Two additional changes at the **e** and **g** positions and five changes at the non-interacting **f** position were made to add chromophores or to increase the helical propensity or the net charge of the designed peptide.

Peptide expression, synthesis and purification

APC-55, a peptide corresponding to amino acids 2–55 of human APC, was produced by expression in *E. coli* [36]. Cell pellets resuspended in 80 mM Tris HCl pH 8.0, 0.2 mM EDTA, 20 mM KCl were lysed by

sonication in a dry ice/ethanol bath. The pH was lowered to 2 by dropwise addition of concentrated HCl, and the acidified lysate was cleared by centrifugation. The supernatant was neutralized, diluted twofold and loaded onto DEAE cellulose. The column was developed with 10–1000 mM KCl. The peak fractions of peptide were concentrated and purified by reversed-phase high performance liquid chromatography (HPLC) [36]. Purity and amino-acid composition of the peptide were confirmed by electrospray mass spectrometry.

Complementary peptides were synthesized with Fmoc chemistry using an Applied Biosystems 431A peptide synthesizer. Met residues in anti-APCp1 were replaced with isosteric norleucine (Nle) to prevent side-chain oxidation. Anti-APCp1 conjugated to biotin contains Met instead of Nle, an additional Leu8Val mutation and Glu–Asp–Glu (wild-type APC sequence) at the peptide carboxyl terminus. This probe was indistinguishable from anti-APCp1 in native gel experiments and CD experiments (data not shown). The anti-APCp1 peptide was biotinylated on the resin with succinimidyl-6-(biotinamido) hexanoate (Pierce) in dimethylformamide. Peptides were purified by reversed-phase HPLC using the same column and gradient as for the wild-type sequence. The identity and purity of all peptides were assessed by electrospray mass spectrometry; all masses measured were within 0.3 atomic mass units of expected values.

Circular dichroism (CD) measurements

CD spectra were recorded in a 1 mm pathlength cuvette at 5°C using an Aviv 62DS circular dichroism spectrophotometer. Peptide stocks were diluted in 10 mM KPO_4, 100 mM KF, pH 7.0. Thermal denaturations were carried out in steps of 1–3°C with 2 min for equilibration and 1 min for data averaging. Thermal transitions were > 90% reversible. Apparent T_m was calculated as the maximum of the first derivative of the CD signal at 222 nm with respect to T^{-1}.

Equilibrium sedimentation

Ultracentrifugation experiments were carried out using a Beckman X-LA ultracentrifuge. Absorbance was monitored at 215, 230 and 280 nm at peptide concentrations of 75, 150 and 250 μM in 10 mM KPO_4, 100 mM KF, pH 7.0. Apparent molecular weights were calculated using the program HID4000 [51] by simultaneously fitting the three data sets recorded at different wavelengths.

Native gel electrophoresis

Samples of the individual peptides and an equimolar mixture of the peptides were prepared and diluted twofold in 0.2% (weight : volume) methyl green, 20% glycerol, 750 mM β-alanine acetate pH 6.0. The peptides (9.0 μg) were loaded at a total peptide concentration of 600 μM. Gels contained 7.5% acrylamide in 375 mM β-alanine acetate, pH 4.0. Samples were run for 2 h at 100 volts, and the gels were fixed with 2% glutaraldehyde and stained for at least 1 h in 0.2% Coomassie brilliant blue in 50% methanol and 10% acetic acid (volume : volume). Destaining was carried out overnight in the same solvent lacking the dye.

Western blotting

Lysates of *E. coli* expressing GST alone or the GST–APC-55 polypeptide and pellets from lysates containing insoluble GST–APC-373 [36] or GST–anti-APCp1 polypeptides were adjusted to 1 mg/ml and diluted 10-fold in lysates from induced cells containing no APC sequences. Each sample (5 μg) was electrophoresed on a 10% Tricine–SDS polyacrylamide gel [52] and transferred to a 0.45 micron nitrocellulose. Blots probed with anti-APCp1 were blocked for 1 h in PNT buffer (10 mM phosphate, pH 8.0, 75 mM NaCl, 0.1% Tween-20) containing 5% bovine serum albumin (BSA) and incubated for 1 h at 42°C with 5 nM biotinylated anti-APCp1 peptide in PNT buffer containing 1% BSA. After 30 min at room temperature in streptavidin-conjugated horseradish peroxidase (HRP) diluted 1:2000 in PNT, the blots were washed three times for 15 min in PNT buffer and developed using enhanced chemiluminescence (ECL) substrate (Amersham). Blots probed with an anti-APC antibody were blocked for 1 h in TBST (10 mM Tris pH 7.5, 137 mM NaCl, 0.1% Tween-20) containing 5% BSA and incubated for 1 h at 42°C with anti-APC Ab-1 (1:2000; Oncogene Science/Calbiochem) in TBST buffer containing 1% BSA. After 30 min at room temperature in HRP-conjugated anti-mouse IgG (1:30,000) in TBST, the blots were washed three times for 15 min in TBST buffer and developed as above.

Peptide-mediated and antibody-mediated precipitation and co-precipitation of endogenous APC and β-catenin

APC was precipitated from three different cell lines: 293, which expresses full-length APC; DLD-1, which contains a mutation at codon 1417 and expresses a truncated APC molecule of approximately 190 kDa; and HT-29, which expresses two truncated APC molecules of approximately 100 and 200 kDa [53]. Cells were harvested at 90–100% confluency, washed twice with ice-cold Dulbecco's phosphate buffered saline (D-PBS, Gibco BRL) containing $MgCl_2$ and $CaCl_2$, scraped into 15 ml ice cold D-PBS and pelleted in a Beckman J-6 centrifuge for 5 min at 4°C. Cell pellets were resuspended in 1 ml ice-cold phosphate buffer, pH 8, containing 0.1% NP-40, 40 μg/ml Pefabloc SC (Boehringer Mannheim) and 10 μg/ml each of leupeptin, pepstatin and aprotinin. The suspension was sonicated, then pelleted for 15 min at 4°C in a microcentrifuge.

For peptide-mediated precipitations, 2 nmol biotinylated anti-APCp1, unbiotinylated anti-APCp1, or D-biotin were added on ice to aliquots of cell lysate (aliquots typically contained 0.5–1 mg total protein by Bradford assay). Streptavidin magnetic particles (1 mg; Boehringer Mannheim), preblocked overnight in lysis buffer containing 5% BSA, were added to each reaction and incubated, rocking, for 1 h at 4°C. Pellets were washed five times in ice-cold lysis buffer and incubated for 10 min at 55°C in 10 μl PBS containing 0.1 mM D-biotin. SDS–PAGE sample buffer (10 μl) was added to each pellet and reactions were incubated for 5 min at 95°C.

For antibody-mediated precipitations, 3 μg anti-APC Ab-1 or a non-specific mouse IgG_1 antibody (Sigma) was added. After 30 min on ice, protein-G–agarose (preblocked overnight with 5% BSA in lysis buffer) was added to the lysates and allowed to incubate for 1 h, rocking, at 4°C. Following five washes with ice-cold lysis buffer, protein-G–agarose pellets were resuspended in 15 μl SDS–PAGE sample buffer and incubated for 5 min at 95°C. Precipitates were electrophoresed on a 6% Tris-glycine polyacrylamide gel (Novex), electroblotted to nitrocellulose and probed.

Blots were blocked for 1 h in TBST containing 5% BSA and probed with anti-APC antibody Ab-1, or a monoclonal anti-catenin antibody (Transduction Laboratories). Anti-APC Ab-1 was diluted 1:150 in TBST, 1% BSA and anti-catenin antibody was diluted 1:2000. Blots were washed three times for 5 min each in TBST and incubated for 1 h in HRP-conjugated rabbit anti-mouse IgG_1 antibody (Pierce) diluted 1:30,000 in TBST and 1% BSA. After three 15 min washes in TBST, blots were developed using ECL substrate (Amersham).

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