Identification of a Link between the SAMP Repeats of Adenomatous Polyposis Coli Tumor Suppressor and the Src Homology 3 Domain of DDEF*

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The adenomatous polyposis coli (APC) tumor suppressor protein is a multifunctional protein with a well-characterized role in the Wnt signal transduction pathway and in cytoskeletal regulation. The SAMP repeats region of APC, an Axin-binding site, is known to be important for tumor suppression and for the developmental function of APC. We performed a yeast two-hybrid screening using the first SAMP motif-containing region of Xenopus APC as bait and obtained several SAMP binding candidates including DDEF2 (development and differentiation enhancing factor 2), which is an ADP-ribosylation factor (Arf) GTPase-activating protein (GAP) involved in the regulation of focal adhesions. In vitro and in cells the Src homology 3 (SH3) domain of DDEF2 and its close homolog, DDEF1, are associated with the SAMP motif of APC competitively with Axin1. Moreover, NMR chemical shift perturbation experiments revealed that the SAMP motif interacts at the same surface of the SH3 domain of DDEF as the known SH3 binding motif, PXXP. When fluorescent protein-tagged APC and DDEF are expressed in Xenopus A6 cells, co-localization at microtubule ends is observed. Overexpression and RNA interference experiments indicate that APC and DDEFs cooperatively regulate the distributions of microtubules and focal adhesions. Our findings reveal that the SAMP motif of APC specifically binds to the SH3 domains of DDEFs, providing new insights into the functions of APC in cell migration.

The APC gene product, APC protein, is a microtubule (MT)-associated protein that accumulates specifically at the plus ends of MTs and is, thus, categorized as a "plus-end-tracking protein" or as a "+TIP" (1, 2). This class of proteins, showing an intriguing accumulation at growing MT plus ends, consists of diverse molecules having different structural properties, including EB1 (end-binding 1), CLIPs (cytoplasmic linker proteins), CLASPs (CLIP-associated proteins), dynein, and dynactin (3–7).

To date +TIPs have been proven to play an important role in the polarized organization of MT networks. So far, some of +TIPs such as APC, ACF7, and CLASPs were reported to be associated with the cell cortex near the migrating edges of cells and to stabilize MTs (3, 8–11). Without the activities of these cortical +TIPs, in most cases the MT stabilizing effect at the leading edges is lost, and the cells fail to maintain polarized, coordinated migration in response to monolayer wounding (9, 12, 13).

In the front of a motile fibroblast, the transient interaction of the plus-end of MT with focal adhesions (FAs) promotes adhesion disassembly and remodeling of the actin cytoskeleton (14). Indeed, MTs are involved in the disassembly of FAs via focal adhesion kinase (FAK) and dynamin (14, 15). On the other hand, paxillin, a FA adaptor protein involved in FA dynamics, regulates MT behaviors by promoting the disassembly of MTs (16). However, the factor(s) and the precise molecular mechanism(s) connecting the MT plus end and FAs are largely unknown. In this context, it is interesting to examine the involvement of APC in the FA dynamics.

The APC protein is a large, 310-kDa protein with multiple structural domains and multiple binding partners, as shown in Fig. 1A (17). In the NH2-terminal armadillo repeat region, APC associates with the kinesin motor protein via KAP3 to transport the molecule to MT plus ends (18). In the COOH-terminal region, APC directly associates with MTs and with MT-binding proteins of the EB1 (end-binding 1) family (19–22). APC stabilizes MTs (23) and promotes the net growth of MTs in cells (24). The middle part of APC protein, which contains 15-amino acid (aa) repeats and distinct 20-aa repeats, directly interacts with β- or γ-catenin (25, 26). The 20-aa repeat region of APC contains 3 repeats of a Ser-Ala-Met-Pro (SAMP) motif, which binds to Axin1/conductin (27, 28). These repeats, initially termed SAMP repeats because of the SAMP motifs found in human APC (27), contain a conserved sequence (I/L)XXXCL-XXMX(K/R) (where X is any amino acid) where (I/L)XXXCL also appears to be invariant in Drosophilia APC (29). Through this middle portion, APC forms a complex with catenins, Axin1/conductin, and glycogen synthase kinase-3β, an important functional unit in the Wnt signaling pathway (25, 30).
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A rat Axin1 cDNA (rAxin1) was a gift from Dr. A. Kikuchi (Hiroshima University). A X. laevis Axin1 cDNA (BC089276, IMAGE:3379167) and a human vinculin cDNA were obtained from Open Biosystems and OriGene, respectively. To generate GFP or mRFP fusion proteins, Axin1, rAxin1, vinculin, and candidates for SAMP-binding proteins were subcloned into pEGFP or pmRFP expression vectors. The details are provided in the supplemental materials.

To generate recombinant proteins in Escherichia coli, PCR-engineered fragments were inserted into the pET32a (Novagen) or pGEX-5X-1 (GE Healthcare) plasmids. The fragments used were as follows: Axin1-RGS, aa 88–211 of X. laevis Axin1; DDEF1-SH3, 73 aa from the stop codon of X. laevis DDEF1; DDEF2-SH3, aa 958–1029 of X. laevis DDEF2; ARHGAP10-SH3, aa 711–783 of X. laevis ARHGAP10; ARHGAP26-SH3, aa 700–771 of X. laevis ARHGAP26; SAMP#1, 1565–1594 of X. laevis APC; SAMP#2, 1712–1745 of X. laevis APC; SAMP#3, aa 2027–2054 of X. laevis APC. See also supplemental Fig. 4. The RGS fragment from human Axin1 (hAxin1-RGS) was constructed as described previously (37). The recombinant proteins were expressed as fusion proteins with thioredoxin His6 and S tags (Trx-S-tagged) or with GST and purified using nickel-nitrilotriacetic acid-agarose (Qiagen) or glutathione-Sepharose (GE Healthcare), respectively. For NMR studies, uniformly 15N ([13C/15N]-labeled) proteins were expressed by growing E. coli cells in an M9 minimal medium supplemented with [12C6]-D-glucose/[12C3]-glycerol([U-13C6]-D-glucose) and 15NH4Cl as carbon and nitrogen sources, respectively. After purification using affinity chromatography as described above, the recombinant fusion proteins were treated with an appropriate enzyme (recombinant Enterokinase or Factor-Xa (both purchased from Novagen) to separate thioredoxin or GST. The proteins were further purified by anion exchange (HiTrap Q HP (GE Healthcare)) and gel-filtration (HiLoad 26/60 Superdex 75 pg (GE Healthcare)) chromatographies. Chemically synthetic peptides for the FLAG-tagged SAMP motif (FLAG-SAMP#n) and FAK-SII (38) were generated by PEPTIDE Institute Inc. (Japan). The amino acid sequences used are represented in supplemental Fig. 4.

Antibodies and Reagents—Antibodies were raised against the following recombinant proteins expressed using pET32a vectors in E. coli BL21(DE3) (Novagen) and are listed as the antibody specification followed by the aa position in the immunogen: rat anti-human APC monoclonal antibody (mAb) (anti-hAPC-N (#21)), aa 1–282 of human APC; rabbit anti-human APC pAb (anti-hAPC-N), aa 1–282 of human APC; rabbit anti-Xenopus APC pAb (anti-xAPC-N), aa 1–284 of Xenopus APC; anti-Xenopus DDEF2 pAb (anti-xDDEF2-C) aa 709–308 of Xenopus DDEF2. Antibodies generated in rabbits or rats were purified by affinity chromatography (Sunplanet Co., Ltd.). The characterization of the antibodies used in this study is described in the supplemental materials. Purified rabbit anti-AMAP1 (DDEF1) pAb and rabbit anti-AMAP2 pAb and anti-KAP3 (C2) pAb were kindly provided by Dr. Sabe (Osaka Bioscience Institute) and by Dr. Akiyama (Tokyo University), respectively. Mouse anti-APF mAb (Wako), rabbit anti-GFP pAb (Chemicon), chicken anti-GFP pAb (Chemicon), rabbit anti-DsRed pAb

which leads β-catenin to the proteasome-mediated degradation pathway (31).

The SAMP repeat region is thought to be critical in the developmental function and tumor-suppressing activity of APC. During the progression of sporadic colorectal tumors as well as in patients with familial adenomatous polyposis (FAP), mutations in the APC gene result in a roughly half-sized APC protein truncated upstream of the SAMP repeats region (for reviews, see Refs. 32 and 33). The importance of the first SAMP motif has been proven using mouse models carrying targeted mutations in APC (34). Truncations of APC upstream of the SAMP repeats (e.g. Apc1638Δn and Apc1572T) have been shown to be associated with tumorigenesis in heterozygotes and embryonic lethal phenotype in homozygotes. In contrast and importantly, homozygosity for Apc1638Δn, in which mutated APC produces a truncated APC protein retaining the first SAMP motif but missing all of the COOH-terminal domains, is compatible with postnatal life and Apc1638Δn/Apc1638Δn animals do not show any increased tumor susceptibility. To date, the function of the SAMP repeats has been discussed only in the terms of the involvement of Axin1/conductin. However, it is still possible that molecules other than Axin1/conductin bind to the SAMP repeat.

We have analyzed the localization and function of full-length and truncated forms of APC using the A6 cell line, which was established from a normal Xenopus laevis kidney and which can overexpress exogenous APC (11, 35). This trait of A6 cells is advantageous for functional analysis of the APC protein, because in most cultured mammalian cells the expression of exogenous APC is very difficult due to the induction of cell death through apoptosis (36). In the present study we found that the SAMP repeat region has a MT-related function in A6 cells.

By a yeast two-hybrid screening system, we identified DDEF1 (also known as AMAP1, ASAP1, or PAG2) and DDEF2 (also known as AMAP2 or PAG3), paxillin- and FAK-binding proteins, as novel SAMP repeat-interacting proteins, the interaction being mediated through their Src homology 3 (SH3) domains. DDEFs co-localized with APC at the cell edges in a MT-dependent manner when expressed in A6 cells. Through nuclear magnetic resonance (NMR) chemical shift perturbation experiments, we further found that the SAMP motif interacts with the same region of the SH3 domains of DDEFs as the known SH3 binding motif PXXP. Finally, we found that APC and DDEFs cooperatively regulate microtubules and FAs through the SAMP repeats region.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Recombinant Proteins—Enhanced green fluorescent protein expression vectors (pEGFP-C series) were purchased from Clontech (TaKaRa). To generate mRFP1 expression vectors, the GFP coding sequences of pEGFP-C were substituted with mRFP1 (a gift from Dr. R. Tsien). The GFP-fused X. laevis APC protein constructs, fAPC-GFP, GFP-fAPC, nAPC-GFP, ∆APC-GFP, and GFP-cAPC, are described previously (11, 35). Other expression plasmids encoding a series of GFP-fused mutated APC proteins were generated using the GFP-fAPC plasmid. The details are provided in the supplemental materials.
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A

Xenopus APC

KAP3/KIF3A-KIF3B
armadillo repeats
alginogenization
PP2A B56 subunit
PP3A

β-catenin, GSK-3β
fatty acid
microtubule
HDAC, PTP13

Accumulation to cell ends

2019 +

2019 +

nAPC-GFP

2019 +

ΔAPC-GFP

2019 +

mAPC-GFP

2019 +

GFP-cAPC

2019 +

GFP-APC(Δarm)

2019 +

GFP-APC(ΔSAMP)

2019 +

GFP-APC(Δmid)

2019 +

GFP-APC(ΔmidΔEB)

2019 +

GFP-APC(ΔMT)

2019 +

B

iAPC-GFP

2019 +

GFP-iAPC

2019 +

nAPC-GFP

2019 +

ΔAPC-GFP

2019 +

mAPC-GFP

2019 +

GFP-cAPC

2019 +

GFP-APC(Δarm)

2019 +

GFP-APC(ΔSAMP)

2019 +

GFP-APC(Δmid)

2019 +

GFP-APC(ΔmidΔEB)

2019 +

GFP-APC(ΔMT)

2019 +

C

GFP-APC(Δ1060)

2019 +

GFP-APC(Δ1314)

2019 +

GFP-APC(Δ1574)

2019 +

GFP-APC(Δ1641)

2019 +

D

FIGURE 1. Domain structure of APC and localization of mutant APC proteins in Xenopus A6 cells. A, the domain composition of X. laevis APC (black letters) and its binding partners (red letters). B and C, GFP-fused APC proteins used to analyze subcellular distribution. The results are summarized to the right. D, the localization of full-length and of mutated APC proteins in Xenopus A6 cells. The cells were fixed and stained for GFP (green) and tubulin (MTs, red). Only typical examples are shown. See also supplemental Fig. 1. Bars, 20 μm.

(Santa Cruz), mouse anti-vinculin (Sigma), and mouse anti-GM130 mAb (Transduction Laboratories) were purchased from the indicated sources. As secondary antibodies, Cy2- or fluorescein isothiocyanate-conjugated anti-mouse IgG, anti-rabbit IgG and anti-chicken pAbs, rhodamine Red-X or Texas Red-conjugated anti-mouse IgG and anti-rabbit IgG pAbs, and Cy5-conjugated anti-mouse IgG, anti-rabbit IgG and anti-rat IgG pAbs were purchased from Jackson ImmunoResearch.

Cell Culture and Transfection of Plasmids and siRNAs—A6 cells, established from a normal X. laevis kidney, were grown at 23 °C without CO₃ in Leibovitz’s L-15 medium (50% L-15 medium, 10 mM Hepes (pH 7.4), 40% distilled water, 10% fetal bovine serum, penicillin-streptomycin solution). Human kidney epithelial cell line HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C under a 5% CO₂ atmosphere. Non-cancer human mammary epithelial cell line MCF10A cells (ATCC) were grown in mammary epithelial cell growth medium (Sanko-junyaku) supplemented with 100 ng/ml cholera toxin at 37 °C under a 5% CO₂ atmosphere.

Transfections were carried out using the Effectene transfection reagent (Qiagen) for plasmids or HiPerFect transfection reagent (Qiagen) for siRNA according to the manufacturer’s instructions. Drug-resistant A6 cell transfectant clones were selected in the presence of 0.8 mg/ml G418 (Calbiochem) and screened by detecting GFP signals under a fluorescence microscope followed by Western blot analysis. The following A6 stable transfectant clones were used in this study:

A6/GFP-fAPC, clone B5 (11); A6/GFP-APC(Δ1574), clone A6C3; A6/GFP-APC(Δ1641), clone A1; A6/GFP-APC(ΔSAMP), clone 8; A6/GFP-APC(ΔDEF2), clone A4; A6/GFP-rAxin1, clone B4D2. For GFP-rAxin1, due to its toxic effect, only clones with very low expression levels were obtained. Moreover, the expression of GFP-rAxin1 tends to be lost during the serial subcultivation;
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A Xenopus and mammalian SAMP#1 sequences, used for Y2H screening as a bait

B

C

FIGURE 2. Identification of SAMP motif-binding proteins. A, the Xenopus APC fragment used for yeast two-hybrid screening and the corresponding regions of human and mouse APC. The SAMP (Ser-Ala-Met-Pro) motif is indicated with dots. B, the binding site to the prey proteins was narrowed down by yeast two-hybrid β-galactosidase activity detection using APC fragments containing the first SAMP motif (Frag. 1) and the last half of APC fragment used for library screening (Frag. 2). The results are indicated to the right. C, coprecipitation (IP) of mRFP-fused SAMP binding candidates with GFP-IAPC immobilized to protein A-Sepharose using anti-GFP mAb. Axin1 (positive control), DDEF1, DDEF2, and AP-2 mu1 subunit were significantly precipitated with GFP-IAPC. WB, Western blot. Input lanes contain 10% of the cell lysate.

therefore, occasionally GFP-positive cells were sorted using a cell sorter.

Synthetic Stealth siRNAs (Invitrogen) were directed against the following target sites: APC, 5'-TCGTTCTGATTCAGATTCCATCCTTTT-3'; DDEF1, 5'-CCCAATTGGAGATTTCGCCTAAT-3'; DDEF2, 5'-CCTAAGCCGGTTGAAGGCCTCTATA-3'. The siRNAs were transfected at a concentration of 100 nM. The siRNA-treated cells were replated 2 days after transfection and used for assays on day 3.

In Vitro Binding Assay, Immunoprecipitation, and Western Blot Analysis—FLAG-SAMP#1 peptides were immobilized on anti-FLAG M2-conjugated agarose beads (Sigma) in a buffer consisting of 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 0.01% Triton X-100, Complete Protease Inhibitor Cocktail (Roche), and 1% bovine serum albumin. Trx-S-tagged recombinant proteins were incubated with the beads in the same buffer for 1 h at a room temperature. After washing with the same buffer, the bound proteins were eluted from the beads by boiling in SDS sample buffer and subjected to SDS-PAGE followed by Western blot analysis.

Immunoprecipitation was performed as follows. First, to prepare soluble cell lysates, cells were briefly washed with phosphate-buffered saline, lysed with buffer A (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 1 mM vanadate, 50 mM NaF, 1 mM lactacystin (Calbiochem), Complete protease inhibitor mixture, phosphatase inhibitor mixture I (Sigma), and phosphatase inhibitor mixture II (Sigma) containing 1% Nonidet P-40) and centrifuged at 13,000 × g for 10 min. Appropriate primary antibodies and protein A-Sepharose (GE Healthcare) were used for immunoprecipitation. Immunoprecipitated samples were analyzed by SDS-PAGE followed by Western blot analysis.

For Western blot analysis, the samples were separated by SDS-PAGE using gels with appropriate concentrations of acrylamide and transferred to polyvinylidene difluoride or to nitrocellulose membranes. After incubation with appropriate antibodies, the bound antibodies were detected with the ECL plus Western blotting detection system (Amer sham Biosciences).

MT Co-sedimentation Assay—Purified bovine tubulin proteins (Cytoskeleton) were polymerized into MTs and stabilized with taxol in BRB80 tubulin polymerization buffer using a standard protocol. Cells were cultured on 10-cm dishes, briefly washed once with phosphate-buffered saline, and lysed with 1 ml of buffer A. The cell lysates were clarified by centrifugation at 100,000 rpm using a TLA-100.3 rotor (Beckman) for 30 min at 4 °C in a Beckman TLA100 ultracentrifuge. The clarified cell lysates were mixed with 10 μM taxol and 0.2 mg of MTs, layered onto a prewarmed 1-ml open-top thick-walled polycarbonate tubes and centrifuged at 70,000 rpm for 10 min at 37 °C in a TLA-100.3 rotor to pellet the MTs. After complete aspiration of the supernatants and cushions, the MT pellets were resuspended in 100 μl of SDS sample buffer.

Immunofluorescence Staining and Fluorescence Microscopy—Cells cultured on coverslips were fixed with 3.7% formaldehyde for 15 min or in methanol-acetone (1:1) at −20 °C for 2 min. After washing with phosphate-buffered saline, cells were permeabilized with 0.1 or 0.5% Triton X-100 and incubated with 10% fetal bovine serum. Samples were then processed for indirect immunostaining using appropriate primary and secondary antibodies. Samples were washed several times and mounted in ProLong anti-fade reagent (Molecular Probes). Images of cells were acquired and analyzed as described previously (11). For live cell imaging, cells were cultured on glass-based dishes (IWAKI) and observed using a DeltaVision Core microscope system (Applied Precision Inc.) equipped with an Olympus IX71 microscope (PlanApo 100×/1.40 NA oil immersion objective. Out-of-focus signals were removed using the deconvolution technique of the DeltaVision system.

NMR Spectroscopy—NMR experiments were performed on a Bruker AV-400 M or on a DRX-500 spectrometer. The sample conditions were 20 mM sodium phosphate (pH 7.4), 50 mM NaCl, 0.02% NaN3, and 10% D2O for the chemical shift perturbation analyses of the proteins and the SAMP peptides (exper-
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NMR spectra for the main-chain resonance assignment of *X. laevis* DDEF1-SH3 were acquired at 298 K using 1.2 mM $^{13}$C/$^{15}$N-labeled DDEF1-SH3 with a Bruker DRX-500 NMR spectrometer equipped with a triple resonance ($^1$H, $^{15}$N, and $^{13}$C) probe head that had self-shielded triple-axis gradient coils. To assign the $^1$H$_{N}$, $^{15}$N, and $^{13}$C resonances, a series of two- and three-dimensional experiments was performed. These were two-dimensional $^1$H,$^{15}$N HSQC three-dimensional HNCO, HN(CA)CO, CBCA(CO)NH, and HNCACB. All the $^{15}$N and $^{13}$C indirect dimensions were acquired by the time proportional phase incrementation (TPPI)-states method, except for the $^{15}$N dimension of CBCA(CO)NH, which was acquired by the sensitivity enhancement and gradient-echo method. All the experiments except for CBCA(CO)NH used the WATERGATE and the water-flip-back methods for suppression of the large water signal. For all experiments, the spectral width of the $^{15}$N dimension was set to 14.5 ppm. The $^1$H carrier was set at the frequency of the residual water resonance (4.773 ppm), and the $^{15}$N, $^{13}$CO, and $^{13}$C carriers were, respectively, set at 123.232, 176.042, and 44.071 ppm. The acquisition times were 27.2 (20), 85.2 (1024), 37.5 (75), and 5.6 ms (35) for the $^{15}$N, $^1$HN, $^{13}$CO, and $^{13}$C dimensions, respectively, and the corresponding numbers of complex sampling points are described in parentheses. All data were processed with the program NMRPipe (39). The resolutions in the $^{15}$N and $^{13}$C dimensions were doubled by linear-prediction before Fourier transformation. The peaks were analyzed with the program Sparky (developed by T. D. Goddard and D. G. Kneller at University of California at San Francisco).

RESULTS

Subcellular Distribution of Truncated APC Proteins—It has been reported that endogenous and GFP-fused full-length APC are localized at the edges of cells in an MT-dependent manner in a wide variety of cell types (Fig. 1B) (24, 35, 40). The kinesin-based active transport via KAP3/KIF3 complex associating with the armadillo region has been shown to be involved in the localization of APC to the cell ends (18). To gain further insight into the APC domains involved in the MT-related function of APC, we
made a series of GFP-fused APC mutants and analyzed their localization in A6 cells as summarized in Fig. 1 and supplemental Fig. 1. Consistent with previous observations, the armadillo repeat domain was indispensable for the MT-plus end accumulation of APC; GFP-APC(Δarm) lacking only the armadillo repeats distributed along the entire MTs. Using the series of APC proteins shown in Fig. 1B, both the middle region and the MT binding basic region were shown to regulate APC localization to the cell ends. Therefore, we further dissected the middle portion as shown in Fig. 1C. In cells, GFP-APC(Δ1060), GFP-APC(Δ1314), and GFP-APC(Δ1574) distributed throughout the cytoplasm similarly to nAPC-GFP (supplemental Fig. 1). In contrast, GFP-APC(Δ1641) containing the first SAMP motif restored the ability to accumulate at the cell ends (see also Fig. 6). The SAMP repeats of APC are known to be an Axin binding region; however, using available anti-Axin antibodies, we could not detect endogenous Axin in the clusters of GFP-APC(Δ1641) at the cell ends using immunofluorescence. Therefore, we hypothesized that molecules other than Axin are associated with the SAMP motif to regulate APC function.

Identification of SAMP Motif-interacting Proteins—To identify SAMP motif-interacting proteins, we performed yeast two-hybrid screening as described previously (41–43). An X. laevis APC fragment containing the first SAMP motif (aa 1565–1644, Fig. 2A) fused to the GAL4 DNA binding domain was used as bait in two-hybrid screens of a mouse brain cDNA library. Approximately 110,000 transformants were screened. Among the colonies that appeared, we isolated 30 clones showing β-galactosidase activity, recovered the plasmids, and analyzed the insert sequences. We identified one clone containing the Axin1 cDNA, seven clones containing the DDEF2 (mKIAA400) cDNA (SH3 domain), one clone containing the ARHGAP26 (mKIAA0621) cDNA (SH3 domain), nine clones containing the FIP200 (mKIAA0203) cDNA, and six clones containing the adaptor protein complex AP-2 mu1 subunit cDNA. Other clones contained inserts encoding untranslated sequences. The endogenous expressions of all these molecules in A6 cells were confirmed by reverse transcription-PCR (data not shown). In the yeast two-hybrid system,
the binding sites on APC were narrowed down to the first half of the fragment used for the library screening, which contains the SAMP motif (Fig. 2B).

To examine the physiological interactions with APC in cells, we performed pulldown assays using A6 cell lysates expressing a large amount of GFP-fAPC (Fig. 2C). GFP-fAPC was immobilized to

![Diagram of DDEF1-SH3 with chemical shift perturbations](image)

**FIGURE 5. Titration of FLAG-SAMP#1 and FLAG-FAK-SII on DDEF1-SH3.** A, the amino acid sequence of DDEF1-SH3 subjected to the NMR analyses. Residues are colored as follows: orange, the SH3 domain of DDEF1; gray, the NH2-terminal nine amino acids derived from the expression vector; cyan, unassigned residues and proline residues with no amide groups. B and C, overlaid spectra of 0.07 mM 15N-labeled DDEF1-SH3 upon the titration of FLAG-SAMP#1 peptide (B) or FLAG-FAK-SII peptide (C). For some resonances the directions and degrees of changes in the chemical shifts are depicted by arrows. D–F, mapping of the residues for which chemical shift changes were observed when FLAG-SAMP#1 (D) or FLAG-FAK-SII (E) was added to the solution of 15N-labeled DDEF1-SH3 on the three-dimensional structure of human Intersectin2-SH3 (PDB coordinate 1UDL), which shows the highest sequence similarity to Xenopus DDEF1-SH3 in the Protein Data Bank (F). Residues are colored according to the scale of the chemical shift changes. D and E were made using PyMol (DeLano Scientific, Palo Alto, CA). The sequence alignment (F) was produced by ClustalW.
In Vitro Binding Analysis of APC-SAMP Motif and SH3 Domains—DDEFs are multidomain proteins containing a pleckstrin homology (PH) domain, an ArfGAP domain, ankyrin repeats (Ank), a proline-rich region, and a COOH-terminal SH3 domain (Fig. 3A). Because in the yeast two-hybrid screen we isolated the SH3 domain of DDEF2 as a SAMP-interacting region, we examined in vitro the direct interactions of the first SAMP motif-containing polypeptide (SAMP#1) with the RGS domain of Axin1 (known as an APC binding region, positive control), the SH3 domains of the DDEFs, and the SH3 domains of ARHGAP26 and ARHGAP10, a close homolog of ARHGAP26, using a synthetic FLAG-tagged first SAMP motif peptide (FLAG-SAMP#1, Fig. 3B) and purified recombinant proteins fused to thioredoxin and S (Trx-S) tags. In vitro pulldown assays revealed that the FLAG-SAMP#1 peptide significantly bound to Axin1 and to the SH3 domains of DDEFs but not to the SH3 domains of ARHGAPs (Fig. 3C). These results demonstrate specific associations between the SAMP motif and the SH3 domains of the DDEFs. Furthermore, the interaction between FLAG-SAMP#1 and Trx-S-Axin1-RGS was disrupted by the addition of GST-DDEF-SH3 in a concentration-dependent manner, showing that the DDEF-SH3 domain and the Axin1-RGS domain competitively bind to SAMP#1 (Fig. 3D).

Analysis of SAMP-SH3 Interaction by NMR Chemical Shift Perturbation—To further confirm the interactions between the SH3 domains and the SAMP motif in vitro, we acquired a series of two-dimensional $^{1}H,^{15}N$ HSQC NMR spectra of $^{15}N$-labeled SH3 domains of DDEFs, ARHGAPs, and human Axin1-RGS upon the addition of a non-labeled SAMP peptide. The sequence of the human Axin1-RGS was the same as that described in a previous structural analysis of APC-SAMP and Axin1-RGS complexes (37). We used a bacterially expressed recombinant peptide as well as a chemically synthesized FLAG-tagged peptide for each of the three SAMP motifs. Two kinds of peptides for each SAMP motif were examined to exclude the possibility of any nonspecific interaction that might be caused by the extrinsic sequence regions. Fig. 4A shows a series of two-dimensional
HSQC spectra of $^{15}$N-labeled human Axin1-RGS with various molar ratios of FLAG-SAMP#1 peptide. As expected, significant chemical shift changes and peak broadening were observed upon the addition of the peptide, confirming that FLAG-SAMP#1 binds to hAxin1-RGS. Likewise, $^1$H,$^{15}$N HSQC spectra of $^{15}$N-labeled DDEF1-SH3 revealed dramatic changes in the presence of FLAG-SAMP#1 compared with spectra in the absence of the peptide (Fig. 4B). In contrast, we observed only marginal differences in the spectra of the $^{15}$N-labeled SH3 domain of Xenopus ARHGAP10 in the absence or in the presence of FLAG-SAMP#1 peptide (Fig. 4C). When FLAG-SAMP#2 or -#3 instead of FLAG-SAMP#1 was added to $^{15}$N-labeled ARHGAP10-SH3, almost no change was observed in the spectra (not shown). To quantitatively estimate the chemical shift perturbations, total chemical shift changes observed when one of the six SAMP peptides was added to one of the $^{15}$N-labeled proteins at an equimolar ratio were calculated (Fig. 4D). We judged that a total chemical shift change of more than 1.0 corresponds to large spectral alteration and can be categorized as a specific interaction. Accordingly, in Fig. 4D, values more than 1.0 are colored blue, and those less than or equal to 1.0 are colored red. Although slight inconsistencies were found in the results in the combinations involving SAMP#3, this is probably due to a problem inherent to FLAG-SAMP#3 rather than to nonspecific binding of recombinant SAMP#3 because we found it difficult to handle FLAG-SAMP#3 peptide in additional experiments. Therefore, the differences in the peptide sequences, including which tag was fused and which SAMP motif was examined, did not affect the corresponding affinities. These results further support the existence of associations between the SH3 domains of DDEFs and APC-SAMP motifs, with affinities that are comparable with those between Axin1-RGS and the repeats. It is noteworthy that in all the combinations of the SH3 domains of DDEFs and the SAMP peptides, the spectra showed similar changes (not shown), indicating that the three SAMP motifs interact on the same region of DDEFs-SH3.

Interaction Site within the DDEF1-SH3 Domain for the APC-SAMP Motif—It is well known that the SH3 domain can bind to a proline-rich motif, PXXP; however, in the SAMP repeats, no such typical proline-rich motif is conserved. Therefore, to characterize the interaction between the DDEF1-SH3 domain and the SAMP motif, we analyzed the region of the SH3 domain that binds to FLAG-SAMP#1 and compared that with binding to the second proline-rich motif of FAK (referred to as FAK-SII hereafter), which is known to interact with DDEF1-SH3 and contains the typical proline-rich motif (38). Initially, the backbone amide resonances of DDEF1-SH3 (consisting of 82 amino acids, as shown in Fig. 5A) were assigned except for seven residues including four prolines and two vector-derived NH$_2$-terminal residues and Gly-64, whose chemical shift changes were that very similar in both their directions and degrees to those observed when FLAG-SAMP#1 was added. This comparison suggests that SAMP#1 and FAK-SII bind to DDEF1-SH3 in a similar manner in terms of their interaction sites on the domain, their affinities, the van der Waals interactions, and the hydrogen-bond networks.
The chemical shift changes observed when FLAG-SAMP#1 or FLAG-FAK-SII was added to the solution of 15N-labeled DDEF1-SH3 are mapped onto the surface of the tertiary structure of the fifth SH3 domain of human Intersectin2, which among available structures exhibits the highest sequence similarity to Xenopus DDEF1-SH3 (Fig. 5, D–F). These figures further demonstrate that SAMP#1 interacts in almost the same region of DDEF1-SH3 as FAK-SII, which contains a typical SH3 binding motif. Therefore, DDEF-SH3s are suggested to have a distinct structural feature in the proline-rich motif binding site that can also specifically recognize the SAMP motif.

DDEFs Associate with the First SAMP Motif in A6 Cells—We then examined the interaction between APC-SAMP and DDEFs in cells. When expressed in the cells, both mRFP-fused DDEF1 and DDEF2 co-localized with GFP-APC(H90041641) at the cell edges (Fig. 6, A and B). Moreover, co-localization of endogenous DDEF2 and GFP-fAPC was also detectable (Fig. 6C). Furthermore, when either GFP-APC(Δ1641) or GFP-APC(Δ1574) was immunoprecipitated from A6 cell transfectants, we found that endogenous DDEFs and Axin1 had bound mainly to GFP-APC(Δ1641) as expected (Fig. 6D). The precipitated fraction including DDEF1 was notably more abundant than that including DDEF2. On the other hand, KAP3 and β-catenin, known APC-binding proteins used as positive controls, interacted equally with both APC fragments.

Based on the above observations, the SAMP-interacting proteins were expected to interact with MT systems. Therefore, the association of DDEFs with MTs was examined by a MT co-sedimentation assay. As shown in Fig. 6E, both endogenous DDEF1 and DDEF2 were precipitated with MTs.

**Mutual Regulation of FAs and MTs by APC, DDEFs, and Axin1—**
DDEFs have been shown to bind to focal adhesion proteins such as paxillin and FAK and regulate FA dynamics (44, 45). Transient overexpression of DDEF1 retarded cell

*Figures 8.* Distribution of APC proteins, MTs, and FAs in A6 transfectants. A, parental A6 cells and A6 stable transfectants expressing GFP-fAPC or GFP-APC(ΔSAMP) (green), cultured for 24 h (5 x 10^4 cells/well of 6-well plate), were fixed and stained for α-tubulin (MTs, red) and vinculin (cyan). Insets are the 2.5×-magnified images of the boxed areas. Arrows indicate the APC-positive MTs extending to the end of cells. Note that in GFP-APC(ΔSAMP)-expressing cells, MTs were tangled behind the FA-rich areas. In the bottom, yellow lines indicate the outline of a cell expressing RFP-Axin1. In both cases, the formation of FAs was restricted to the cell periphery. Bars, 10 μm. B, RFP-DDEF1 or RFP-Axin1 was transiently overexpressed in A6 transfectants expressing GFP-fAPC. RFP-DDEF1 was co-localized with GFP-fAPC without disturbing the distribution of GFP-fAPC along MTs. RFP-Axin1 formed granular aggregation with GFP-fAPC and removed it from MTs (arrows). In the bottom, yellow lines indicate the outline of a cell expressing RFP-Axin1. In both cases, the formation of FAs was restricted to the cell periphery. Bars, 20 μm. C, the numbers of FAs visualized with vinculin staining in parental A6 cells and A6 transfectants (indicated in the figure) were counted and plotted in the graph. Results are the means ± S.E. For each condition >60 cells were observed.
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spreading by regulating the function of a subset of focal adhesion proteins including paxillin (38, 46). Consistent in A6 cells, endogenous DDEF1 and DDEF2 are localized at the FAs as well as plasma membranes (data not shown). We examined whether APC affects the focal adhesions through its SAMP repeat. For this purpose we examined the phosphorylation status of paxillin (Tyr-31), which is highly increased during cell adhesion and migration (47) in A6 transfectants overexpressing GFP-fAPC, GFP-APC (ΔSAMP), GFP-rAxin, or GFP-DDEF2. As shown in Fig. 7B, the phosphorylation of paxillin at Tyr-31 was significantly reduced in cells expressing GFP-APC (ΔSAMP), GFP-rAxin, or GFP-DDEF2, whereas the total expression levels of paxillin were not reduced in these cells. In addition, the acetylation of tubulin, an indicator of stable MTs, was reduced in GFP-rAxin- and GFP-DDEF2-expressing cells (Fig. 7C).

To understand these phenomena, we observed the distribution of FAs and MTs in these transfectants by immunofluorescence (Fig. 8). The FAs were visualized with vinculin, a core adhesion component that remains in the adhesions even after paxillin displacement (16). Endogenous APC and GFP-fAPC are distributed along the MTs extending to the extreme edges of cells beyond the FA-reach areas (arrows). The expression of GFP-fAPC produced large number of FAs along the entire basal cell cortex. In contrast, in GFP-APC(ΔSAMP)-expressing cells, limited numbers of FAs were distributed only at the cell margin, and MTs decorated with GFP-APC(ΔSAMP) are tangled behind the FA-rich areas.

The processes of organization of FAs and MTs in these transfectants were visualized by a time-lapse imaging study shortly after cell plating (Fig. 9, supplemental Movie 1). Although GFP-fAPC-positive MTs dynamically extend toward the cell ends (arrows in Fig. 9A), most MTs decorated with GFP-APC(ΔSAMP) turn behind the FAs and cannot get over the FAs. It is striking that the remodeling of FAs is retarded in GFP-APC(ΔSAMP)-expressing cells (Fig. 9B), consistent with the Western blot result showing the significant reduction of the phosphorylation of paxillin (Tyr-31), an indicator of FA dynamics (Fig. 7B). These observations demonstrate the function of the SAMP repeats in MT organization and FA regulation.

Then, to investigate whether APC affects FA dynamics through DDEF or Axin, we transiently overexpressed RFP-Axin1 or RFP-DDEF2 in A6 transfectants expressing GFP-fAPC and analyzed the effect on the FA distribution (Fig. 8, B and C). Although the distribution of GFP-fAPC along MTs was not affected by the overexpression of RFP-DDEFs, which was clearly co-localized with GFP-fAPC (data not shown), the number of FAs was significantly decreased, especially from the central region of cells, similarly to the phenotype observed in the GFP-APC(ΔSAMP)-expressing cells. When RFP-Axin1 was overexpressed, the FA number was also decreased. However, unlike the effect of RFP-DDEF1 overexpression, the distribution of GFP-fAPC was seriously disturbed and removed from MTs by RFP-Axin1. A similar effect of overexpression of Axin1 on the distribution of endogenous APC and FAs was observed in the parental A6 cells (data not shown). Therefore, in the overexpression study, both DDEFs and Axin1 affected MT and FA dynamics probably via APC, but their mechanisms of action are distinct.

Effect of RNA Interference of APC or DDEFs in Non-cancer Cell Line MCF10A—Finally, we confirmed the function of APC and DDEFS on the distribution of microtubules and FAs using an RNA interference strategy in the non-cancer human mammary epithelial cell line MCF10A (Fig. 10A). In APC knocked-down cells, FAs became enlarged at the cell periphery, whereas DDEFS knocked-down cells formed thinner FAs (Fig. 10B). At the peripheral regions of cells, MTs are radially aligned facing their plus ends toward the edges. However, in the DDEF1-concentrated lamellipodia (Fig. 10C, arrowheads), only few MTs decollected with APC extended toward the cell edges, whereas many MTs are terminated behind the DDEF1-rich areas. In APC knocked-down cells, the number of MTs invaded into the DDEF1-rich areas was slightly decreased (Fig. 10D). On the contrary, after DDEF knockdown, large numbers of MTs reached the cell edges in most lamellipodia. These findings suggest that DDEFS suppress the invasion of MTs, whereas APC promotes the growth of MTs, consistent with the observation from the overexpression experiments described above.

DISCUSSION

In the present study, based on the subcellular distribution of a series of engineered APC proteins in Xenopus A6 cells, we
found that, in addition to the armadillo repeats associating with kinesin motor protein and the basic region binding to MTs directly, the SAMP repeats region has a MT-related function, although the molecular mechanism that targets the APC fragment retaining the SAMP repeats is not yet clear. The SAMP repeats of APC have been known to be an Axin binding region, which is involved in the tumor suppressing activity and in the developmental function of APC. Here, we explored the possibility that the SAMP motif binds to molecules other than Axin and found that DDEF1 and DDEF2 interacted with this region through their SH3 domains. In vitro binding assays demonstrated that DDEFs bind to the SAMP motif competitively with Axin1. This interaction was also verified by the NMR chemical shift perturbation study. Although the SH3 domain of ARHGAP26 was also identified as a SAMP binding candidate in yeast two-hybrid screening, in vitro binding assays and NMR studies revealed that the SAMP motif binds selectively to DDEFs-SH3.

The SH3 domains of both DDEF1 and ARHGAP26 have been reported to bind to the SII region of FAK, which contains the typical SH3 binding motif PX...P (38, 48), suggesting that the SH3 domains of these molecules have relatively similar characteristics in the context of protein-protein interaction. Nevertheless, we observed preferred associations of the SAMP motif with the SH3 domains of DDEFs. Thus, these observations suggest a specific association of the SAMP motif with DDEFs, relative to all other SH3-containing proteins, although the possibility of its interaction with other SH3 proteins cannot be excluded.

The SH3 domain is well known to bind to the proline-rich motif PX...P. However, this motif is not observed in any of the three SAMP motifs of APC. Therefore, the mode of interaction between APC-SAMP and DDEFs-SH3 is structurally interesting. A proline-rich sequence encompassing the PX...P motif forms a left-handed polyproline type II (PPII) helical conformation (49). Because, despite its name, such a PPII helix can also be formed by amino acid sequences containing no proline residues, the SAMP motif, containing one proline residue, may also adopt a PPII helix. The PPII helix has a triangular cross-section, and
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residues along two of the three edges interact with the SH3 domain. Because the two prolines in the PXXP motif and their respective preceding residues occupy each of the two edges, these XP pairs usually contribute to the specificity of the interaction (49). Comparing the sequences of the SAMP motif and FAK-SII, both have at least one pair of basic residues and a proline. Therefore, these amino acid arrangements may be involved in binding to the SH3 domains of DDEFs by forming a PPII helix. However, these residues alone could not explain the selectivity of the SAMP motifs for DDEFs over ARHGAPs, because FAK-SII can associate with both of them.

As a possible cause for this selectivity, we have focused on a notable feature of the SAMP motifs; their COOH-terminal parts are rich in arginine and lysine residues. These basic residues may also be involved in favorable electrostatic interactions between the SAMP motif and DDEFs-SH3. This notion is consistent with the presence of basic residues proximal to the PXXP core element, which often govern the orientation on a certain SH3 domain of the proline-rich polypeptide and which can align itself in either one of the two opposite directions because of the pseudo 2-fold symmetry of a PPII helix (50, 51). Another possibility is that the cluster of basic residues is the key determinant of the specific interactions between APC-SAMP and DDEFs-SH3. Indeed, several SH3 domains recognize consensus sequences comprised of arginine and lysine residues, such as (R/K)XX(R/K) (52) and (K/R)XXXKKX(K/R)(K/R) (53). Considering that three SAMP motifs interact with DDEFs, it is plausible that both the positively charged residues and the conserved residues including Ser-Ala-Met-Pro are necessary for the specific binding to the SH3 domains. Although the crystal structure of Axin1-SAMP complex has already revealed that the NH2-terminal part of the SAMP motif has propensity to form an α-helix (37), the conformation of the basic residue-abundant COOH terminus, which we expect to be important for the binding to DDEFs-SH3 for the above reason, is not obvious. To elucidate the structural basis of the specific interaction between APC-SAMP and DDEFs-SH3, we are determining the detailed structure of the complex.

DDEFs have been reported to bind, not only to FAK, but also to other focal adhesion related proteins, including paxillin (44, 45, 54) and to endocytic proteins such as amphiphysin II (53). DDEFs are phosphatidylinositol 4,5-bisphosphate-dependent ArfGAPs and are reported to exhibit GAP activity toward Arf1 and Arf6 (55, 56). Through their Arf GTPase-activating activity, DDEFs regulate membrane trafficking and the actin-based structures such as FAs and thereby regulate cell movement (46, 57). The NMR study demonstrated that SAMP#1 interacts in almost the same region of DDEF1-SH3 as FAK-SII, suggesting that the binding of APC to DDEFs could compete with FAK. Considering the fact that APC is involved in cell migration at the leading edges of cells (11, 58, 59), APC was expected to affect FAs through a complex with DDEFs. Indeed, we found that the overexpression of APC enhanced FA turnover relying on the SAMP repeats region, and this effect was suppressed by the overexpression of DDEFs. These results suggest that the balance between the activities of APC and DDEFs cooperatively regulate the FA dynamics, although it is still possible that DDEFs compete with other SAMP-binding proteins having a function in FA regulation. Moreover, because the GFP-APC(ASAMP) construct lacks a large portion including the SAMP repeats and 6 of 7 20-aa repeats, it is possible that other molecules contribute to APC function by interacting with deleted regions outside of the SAMP motifs. Nevertheless, the alteration of FA-regulating function of APC caused by the loss of DDEF binding ability could be explained by the cell spreading activating and suppressing functions of APC and DDEFs, respectively. This hypothesis fits with the model that a subset of MT plus ends decorated with APC promotes FA remodeling at the migrating edges of cells (14, 16). Therefore, future studies must investigate the detailed molecular mechanisms of cooperation between APC and DDEFs, possibly through the Arf signaling.

At the same time we found an unexpected effect of the overexpression of Axin1 on APC distribution and function; when overexpressed, RFP-Axin1 formed granular clusters with APC in the cytoplasm and removed APC from MT ends. Under these conditions, FA assembly was restrained, consistent with the notion that APC could accelerate the formation of FAs. However, in cells under normal conditions, the expression level of Axin is much lower than that of APC (60). In most cells expressing Axin endogenously, APC can be localized at MT ends; therefore, at the appropriate expression levels Axin may not exert such a drastic effect on APC localization. It is possible that Axin could moderately accelerate the turnover of APC to remodel the MT networks.

Previous studies focused on the involvement of Axin to explain the importance of the SAMP repeats of APC in its developmental and in its tumor suppressing function. However, this study has shown that molecules other than Axin, such as DDEFs, can contribute to regulation of the activity of APC. In this study we could not clarify the meaning of the competition between DDEFs and Axin for APC binding solely from the assays using cultured cells in vitro. An alternative explanation is that DDEFs do not directly compete with Axin, and both molecules simultaneously bind to the SAMP motifs that repeat three times at the middle portion of APC under physiological

![Figure 10](image-url) - siRNA-mediated inhibition of APC or DDEFs affects FAs and MTs in the human mammary epithelial cell line MCF10A. A, characterization of siRNAs. Total lysates of MCF10A cells, transfected with the indicated siRNAs, were prepared 72 h after transfection and separated by SDS-PAGE in 4 or 7.5% gels for APC or DDEFs, respectively, and subjected to Western blotting (WB) with the indicated antibodies. Dilution series (100, 50, and 20%) of Mock samples was also loaded. The levels of APC DDEF1 and DDEF2 were reduced by ~70%. B, effect of APC or DDEFs knockdown on the distribution of FA visualized with vinculin. The cells transfected with the indicated siRNAs were fixed and stained for vinculin 72 h after transfection. Note that in APC knocked-down cells, FAs became larger at the cell periphery, whereas DDEFs knocked-down cells formed thinner FAs. Bars, 10 μm. C, distribution of MTs in the lamellipodia. The cells transfected with the indicated siRNAs were fixed and stained for α-tubulin (MTs, green), DDEF1 (cyan), and APC (red) 72 h after transfection. The yellow lines indicate the edges of lamellipodia. Occasionally DDEFs are concentrated in a part of lamellipodia (arrowheads), whereas only few MTs decorated with APC reached the edges of cells. In DDEFs knocked-down cells, many MTs reached to the cell edge. Insets are 2× zoom of the boxed areas. Only the microtubule channel (green) and APC channel (red) are superimposed. Bars, 10 μm. D, the number of MTs in a 2 × 5 μm area (yellow box) from the cell edge was counted and summarized in the table. For Mock and APC kinase dead (KD) samples, DDEF1-concentrated regions were analyzed. RNAi, RNA interference.
conditions. To gain insight into the physiological meaning of the association of DDEFS and Axin with APC, we need to perform more refined experiments in mammalian cells and on animals. Moreover, although in the present study we have not examined a possible involvement of DDEFS in the Wnt signaling pathway, undoubtedly this is of great importance for future study. In this connection it is interesting that the inhibition of ArfGAP activities in cells by a small molecule compound modulated Wnt/β-catenin signaling (61). We expect that the evaluation of these possibilities will provide us with further understanding of the molecular mechanisms of the tumor suppressing function of APC.

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