APC mutations in colorectal tumours from FAP patients are selected for CtBP-mediated oligomerization of truncated APC

Jean Schneikert*, Katharina Brauburger and Jürgen Behrens

Nikolaus-Fiebiger-Center for Molecular Medicine, University of Erlangen-Nürnberg, Glückstrasse 6, 91054 Erlangen, Germany

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The germline transmission of a mutation in the *adenomatous polyposis coli* (*APC*) gene leads to cancer of the gastro-intestinal tract upon somatic inactivation of the remaining allele in familial adenomatous polyposis (FAP) patients. *APC* mutations result in truncated products that have primarily lost the ability to properly regulate the level of the transcription factor β-catenin. However, colorectal cancer cells from FAP patients always retain a truncated APC product and the reasons for this strong selective pressure are not understood. We describe here the surprising property for the transcriptional repressor C-terminal binding protein (CtBP) to promote the oligomerization of truncated APC through binding to the 15 amino acid repeats of truncated APC. CtBP can bind to either first, third or fourth 15 amino acid repeats, but not to the second. CtBP-mediated oligomerization requires both dimerization domains of truncated APC as well as CtBP dimerization. The analysis of the position of the mutations along the *APC* sequence in adenomas from FAP patients reveals that the presence of the first 15 amino acid repeat is almost always selected in the resulting truncated APC product. This suggests that the sensitivity of truncated APC to oligomerization by CtBP constitutes an essential facet of tumour development.

INTRODUCTION

Familial adenomatous polyposis (FAP) is a dominantly inherited genetic disease characterized by the germline transmission of a truncating mutation in the *adenomatous polyposis coli* (*APC*) gene (1,2). FAP patients develop hundreds to thousands of intestinal polyps upon somatic inactivation of the second *APC* allele that evolve into aggressive tumours if left untreated. It is thought that sporadic colorectal cancer recapitulates FAP, involving disease initiating, somatic biallelic *APC* mutations in the vast majority of cases (3).

The *APC* gene (4) encodes a huge protein of 2843 amino acids that has been implicated in various cellular functions (5–10). However, *APC* is best understood as a negative regulator of the transcription factor β-catenin, the effector of the canonical wnt signalling pathway (11) that controls the coordinated expansion and differentiation of the intestinal crypt stem cells (12,13). In the absence of a wnt signal, β-catenin is recruited in the so-called destruction complex (14) comprising the core components axin/conductin (15), casein kinase 1 and glycogen synthase kinase 3β (GSK3β). This results in β-catenin phosphorylation and its subsequent degradation in the proteasome (16–18). In the presence of a wnt signal, the destruction complex is inactivated and stabilized β-catenin associates with transcription factors of the lymphoid enhancer factor-1/T-cell factor family (19) to activate cell proliferation through the regulation of a set of target genes.

*APC* gene mutations in colon cancer are either point mutations or small deletions and insertions leading to stop codons and therefore to truncations of the protein (20–23) that occur early during tumorigenesis (24). Alternatively, one allele may be lost by mitotic recombination, leading to loss of heterozygosity (LOH) (25,26). One important consequence of *APC* truncation is the removal of all axin/conductin binding sites (the so-called SAMP repeats), often the β-catenin inhibitory domain (CID) (27) and most, β-catenin binding, 20 amino acid repeats (Fig. 1). This results in β-catenin stabilization (28) and enhanced β-catenin-dependent transcriptional activity that drives constitutive cell proliferation, mimicking the effects of permanent wnt stimulation.

*To whom correspondence should be addressed. Tel: +49 91318529114; Fax: +49 91318529111; Email: jschneik@molmed.uni-erlangen.de

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APC mutations are not random and the position of the germ-line mutation in FAP patients determines the nature and the site of the somatic hit (29–32). Accordingly, there is a very strong selection for mutations leading to the retention of a truncated APC product of a minimal length, likely fulfilling an essential function (33). When negative and positive selections are combined, i.e. stabilization of β-catenin versus retention of a minimal fragment, one realizes that APC mutations have the tendency to concentrate between the end of the 15RA and the middle of 20R3, thereby defining the so-called mutation cluster region (22,34,35) (Fig. 1).

A component interacting with the C-terminal end preserved in truncated APC may represent an essential target of the positive selection of APC mutations, important for tumour development. Potential candidates include the C-terminal binding protein 1 (CtBP1), a transcriptional repressor originally identified as an interacting partner of the adenovirus E1A oncogene (36). The mammalian genome contains two CtBP genes, CtBP1 (37) and CtBP2 (38), that are both β-catenin target genes up-regulated in colorectal tumours (39–41). It has been reported that truncations of APC abolish its ability to target CtBP1 for degradation (41) and it is currently discussed whether CtBP1 plays a role in adenoma initiation (42,43). CtBP1 contributes also to the regulation of the expression of β-catenin target genes (44–46). CtBP1 has been shown to bind directly to the 15 amino acid repeats (15R) of APC in vitro (47) and we have investigated this interaction in more detail in this study.

RESULTS
CtBP1 and CtBP2 interact with APC via the 15 amino acid repeats

Two independent reports have shown that CtBP1 can interact with full-length APC (45,47). We first asked whether the close relative CtBP2 also interacts with APC, by performing co-localization experiments in the colorectal cancer cell line SW480. In these cells, transiently expressed full-length APC fused at its N-terminus to YFP (yAPC) is found at the microtubule...
cytoskeleton (48), as reflected by the fibre-like appearance of the fluorescence (Fig. 2A). In contrast, the intracellular localization of transiently expressed N-terminally flag-tagged CtBP1 (fCtBP1) is mainly nuclear, whereas fCtBP2 (49) is found exclusively in the nucleus and fCtBP2RR (49), a mutant of fCtBP2 defective in dimerization (see below), distributes like fCtBP1. When coexpressed with yAPC, fCtBP1 and fCtBP2 colocalize with APC at the microtubule fibres, in contrast to the mutant fCtBP2RR that provides a specificity control thereby. We concluded that fCtBP1 and fCtBP2 are likely to interact with yAPC in a similar manner. We reached the same conclusion when transient transfections were performed in DLD1 cells (Supplementary Material, Fig. S1).

The two studies mentioned above (45,47) investigated also the possibility of an interaction of CtBP1 with truncated APC from colorectal cancer cells, but only one reported positively (47). To substantiate the issue, we performed coimmunoprecipitation experiments using antibodies against CtBP1 and CtBP2 in extracts derived from LoVo and HT29 cells expressing different pairs of endogenous APCs truncated at positions 1114/1430 and 853/1555, respectively (Fig. 2B). APCs truncated at either position 1555 or 1430 could be specifically pulled-down together with endogenous CtBP1 and CtBP2, whereas the APC isoform truncated at position 853 was barely detectable in the immunoprecipitate. APC truncated at position 1114 interacted also with CtBP2, but the E12 antibody precluded any clear conclusion about an interaction with CtBP1. We concluded that truncated APC can interact with both CtBP1 and CtBP2, likely requiring for that the domain encompassing residues 853–1114.

To identify precisely the site(s) of CtBP interaction on APC, a series of progressive APC deletions (Fig. 1) was transiently transfected together with a fCtBP1 expression vector into 293T cells. Immunoprecipitation with an anti-flag antibody revealed that APC residues 1020–1060 were necessary for the interaction (Fig. 3A, compare lanes 8 and 9). As this region contains the first 15 amino acid repeat (Fig. 1), we further pursued our analysis using an APC construct truncated at position 1466 and displaying three amino acid substitutions in either one or more 15 amino acid repeats (35) (Fig. 3B, Supplementary Material, Fig. S2). The experiment indicated that mutating successively the first (15RA), third (15RC) and fourth (15RD) amino acid repeats affected significantly the binding of fCtBP1 to APC (Fig. 3B, compare lanes 3, 4, 6 and 7). In contrast, mutating the second (15RB) amino acid repeat (compare lanes 4 and 5) or introducing point mutations in the first 20 amino acid repeat (20R1) that drastically reduce its affinity for β-catenin (35) (compare lanes 3 and 10) had no effect. Finally, a construct containing only wild-type 15RA was binding to fCtBP1 (lane 8), whereas another one displaying only wild-type 15RB was not (lane 9). We concluded that fCtBP1 interacts with the 15RA, 15RC and 15RD of APC.

In the next step, we attempted to delineate other APC domains that might be involved in CtBP1 binding, using a variety of APC fragments (Fig. 3C). We found that the two constructs containing the N-terminal residues 1–205 (lanes 3 and 10) were binding more efficiently to fCtBP1 than any other lacking them, when normalized to the different expression levels (Fig. 3C, Supplementary Material, Fig. S3). Further immunoprecipitation experiments revealed
that the anti-APC antibody Ali failed to pull-down CtBP1, although it was able to coimmunoprecipitate β-catenin (Supplementary Material, Fig. S4A), whereas an N-terminally flag-tagged APC construct immunoprecipitated with an anti-flag antibody could bind both β-catenin and CtBP1 (Supplementary Material, Fig. S4B). These latter observations suggested that the epitope recognized by the Ali antibody, i.e. amino acids 45–140 (50) (http://www.abcam.com/APC-antibody-ALi-12-28-ab58.html last accessed 2011-06-15), is involved in binding of fCtBP1 to APC. We also investigated the influence of the second dimerization domain of APC (51) on CtBP1 binding. The deletion of residues 756–839 or 756–1144 (Fig. 1) resulted in fragments that were binding to CtBP1 as efficiently as the parental construct yAPC1404 (Supplementary Material, Fig. S5). Together, we found that the N-terminus of APC containing the first dimerization domain (52) facilitates the CtBP1–APC interaction.

CtBP1 promotes and stimulates dot formation by truncated APC

Truncated APC constructs containing the CID (27), as exemplified by yAPC1641, yAPC1485 or yAPC1466 locate diffusely in the cytoplasm (Fig. 4, Supplementary Material, Fig. S6). We found that co-expression of fCtBP1 induced the formation of small and round cytoplasmic inclusions by such constructs, so-called dots, in DLD1 (Fig. 4), SW480 (Supplementary Material, Fig. S6) and 293T cells (Supplementary Material, Fig. S7). fCtBP1 co-localized with the inclusions, in line with the results of the coimmunoprecipitation experiments. When the four 15Rs were mutated as in yAPC1466-15Rm to avoid CtBP binding, dot formation was abolished (Fig. 4, Supplementary Material, Fig. S6). To determine the individual contribution of each 15R to CtBP-mediated dot formation, yAPC1466 and mutants thereof containing one or more mutant 15R affected in fCtBP1 binding were expressed together with fCtBP1 in 293T cells and investigated for their ability to form dots (Supplementary Material, Fig. S8A). The experiment revealed that successive mutation of the 15RA–C did not affect the property of fCtBP1 to stimulate dot formation. In contrast, further mutating the remaining 15RD abolished this property (Supplementary Material, Fig. S8A) and the APC construct containing only wild-type 15RB and therefore also unable to bind fCtBP1 did not form dots (unpublished data). We concluded that fCtBP1 stimulates APC dot formation through binding to the 15Rs.

APC constructs lacking the CID such as yAPC1289 formed dots spontaneously (35,53,54) and fCtBP1 co-localized with them (Fig. 4, Supplementary Material, Figs S6, S7 and S8B). The formation of dots and fCtBP1 co-localization were independent of the nature of the tag, since N-terminally flag-tagged APC1287 (fAPC1287) was building inclusions colocalizing with either a gfpCtBP1 (47) or a CtBP2gfp (55) construct (Supplementary Material, Fig. S9). The introduction of point mutations in all four 15Rs in yAPC1289 clearly reduced the proportion of cells displaying dots in the absence of fCtBP1 (Fig. 4, Supplementary Material, Fig. S8B). Western blotting analysis (Supplementary Material, Fig. S8C) showed that this was not resulting from a lower expression level of yAPC1289-15Rm, when compared with yAPC1289. The recruitment of fCtBP1 into the dots did not occur with the 15R-mutated construct yAPC1289-15Rm, indicating that binding of CtBP1 to APC was necessary for colocalization (Fig. 4, Supplementary Material, Figs S6 and S8B). In DLD1 and SW480 cells, the presence of fCtBP1 had no apparent influence on spontaneous dot formation by yAPC1289 (Fig. 4, Supplementary Material, Fig. S6). However, in 293T cells, under conditions of low expression, a stimulating effect of fCtBP1 on dot formation by constructs lacking the CID such as yAPC1404 and yAPC1289 could still be observed, as the fluorescence appearance evolved from a relatively discrete dotty pattern over a diffuse background in the absence of CtBP1 into a pattern made of numerous and bigger dots over a diffuse background of weaker intensity in the presence of fCtBP1 (Supplementary Material, Figs S7 and S8). Under conditions of high APC expression, the latter pattern could also be observed in the absence of fCtBP1.
which was still able to amplify dot formation (Supplementary Material, Fig. S7A). We concluded that the 15R contribute to spontaneous dot formation by constructs lacking the CID, which is enhanced by the presence of CtBP1.

To identify other APC domains responsible for the formation of dots, we used a collection of constructs progressively deleted from the C-terminus (Figs 1 and 5). APCs truncated at position 1060 localized as dots when it was transiently expressed in DLD1 colorectal cancer cells (Fig. 5). Further deletion of the 15RA affected significantly the formation of dots, since yAPC1020 distributed diffusely in the cytoplasm of approximately two-third of the transfected cells. This tendency increased with yAPC950 and reached a maximum with yAPC750 showing almost exclusively a diffuse pattern with only occasionally a few dots. In line with the above results, fCtBP1 co-localized with dots as long as at least the 15RA was present in the construct. When the constructs were transiently expressed in 293T cells at a low level, we made very similar observations (Supplementary Material, Fig. S10). We could force dot formation by yAPC1020 and yAPC950 by allowing expression to proceed for a longer period of time, but even under these conditions, yAPC750 still remained diffusely distributed. Western blot analysis performed under conditions of high expression indicated that fCtBP1 expression resulted in higher levels of APC constructs (Supplementary Material, Fig. S10). However, as yAPC750 levels in the absence of CtBP1 were similar or even higher than the levels of yAPC1247, yAPC1147 and yAPC1060 in the presence of CtBP1, the dots were not simply the consequence of protein accumulation and unspecific aggregation. We concluded that dot formation by truncated APC requires residues 750–1020 that build the second dimerization domain.

**Figure 5.** Dot formation by truncated APC requires the 15Rs and the second dimerization domain. DLD1 cells were transiently transfected with the indicated YFP-APC fusion constructs either in the absence or in the presence of a flag-CtBP1 expression vector. Thirty-six hours post-transfection, cells were stained with an anti-flag antibody and the nuclei were revealed with the Hoechst dye. The percentages indicate the fraction of transfected cells displaying the appearance shown.

**Figure 6.** Spontaneous and CtBP-mediated dot formation require the N-terminus of APC. DLD1 cells were transiently transfected with the indicated YFP-APC fusion constructs either in the absence or in the presence of a flag-CtBP1 expression vector. Thirty-six hours post-transfection, cells were stained with an anti-flag antibody and the nuclei were revealed with the Hoechst dye. The percentages indicate the fraction of transfected cells displaying the appearance shown.
domain of APC (51). The process is facilitated by the additional presence of residues 1020–1060 spanning the 15RA.

Further analysis of APC to reveal domains responsible for the formation of dots was performed with yAPC1404 and N-terminal deletion mutants thereof (Fig. 6, Supplementary Material, Figs S11 and S12). The capacity to build spontaneously dots was lost upon deletion of the first N-terminal 205 residues [compare yAPC1404 and yAPCΔ(1–205)] or the internal residues 756–887 or 756–1144 [compare yAPC1404 with yAPC1404 Δ(756–887) and yAPC1404Δ (756–1144)], encoding the first and second dimerization domain, respectively. Expression of fCtBP1 could rescue the defect, efficiently for yAPC1404Δ(756–887) and yAPC1404Δ (756–1144), but only partially for yAPCΔ(1–205). These results confirmed the importance of the second dimerization domain for spontaneous dot formation and revealed the crucial role of the N-terminal 205 residues of APC for both spontaneous dot formation and stimulation by fCtBP1.

CtBP1 and CtBP2 stimulate dimerization of truncated APC

In the next step, we asked whether CtBP would promote dimerization of truncated APC. To answer this question, we performed coimmunoprecipitation experiments in which fAPC1287 could pull-down yAPC1289 and yAPC1289-15RΔ (Fig. 7A). In the presence of gfpCtBP1, the amount of yAPC1289 bound to fAPC1287 increased substantially (compare lanes 5 and 11), whereas the amount of coimmunoprecipitated yAPC1289-15RΔ remained unchanged (compare lanes 6 and 12). A similar result was obtained with CtBP2gfp (Fig. 7B). We concluded that binding of CtBP to the 15Rs stimulates the dimerization of truncated APC.

The formation of truncated APC dots requires CtBP dimerization

CtBP1 and CtBP2 can homodimerize and heterodimerize (56–58). We thought that this property may favour APC dimerization and dot formation by bridging two APC molecules. Therefore, we took advantage of the availability of the fCtBP2RR construct mutated at amino acid positions 147 and 169 and defective in dimerization (49), what we confirmed in an initial coimmunoprecipitation experiment using both gfpCtBP1 and CtBP2gfp as dimerization partners of fCtBP2RR (Supplementary Material, Fig. S13). Interestingly, fCtBP2RR did interact with truncated APC in a manner dependent on the presence of the 15RA in coimmunoprecipitation experiments (Supplementary Material, Fig. S14), but did not colocalize with truncated APC (Supplementary Material, Fig. S15), similarly to what we observed with full-length yAPC (Fig. 2A). Importantly, fCtBP2RR was deficient in promoting dot formation by yAPC1485 (Fig. 8) that remained diffusely distributed in the cytoplasm. We concluded that APC dot formation requires CtBP dimerization.

CtBP-mediated APC oligomerization correlates with the selection APC mutations

In the last step, we collected the currently available information about the APC mutation spectrum in colorectal tumours from FAP patients (20,29–31). We focused our attention only on the cases where the position and the nature of the mutation were known for both APC alleles. We also restricted our analysis to tumours in which truncated APC does not contain the CID, because it is itself a parameter of the biallelic selection of APC mutations in FAP patients (27). We first observed that out of 227 tumours, 225 contained at least one truncating mutation located after the 15RA (Fig. 9). APC mutations are interdependent, the position of the germline
mutation along the APC sequence determining the nature and the location of the somatic hit in the other allele (32). Accordingly, a germline mutation falling after the 15RA (i.e. from position 1053) is associated with LOH at the second allele in 84% of the cases (Fig. 9). LOH proceeds essentially by homologous recombination (25,26) which is spontaneously more frequent than any other mutational event (59). Thus, LOH indicates that there is no selective pressure for the acquisition of a point mutation in the second allele and therefore that the truncated APC product resulting from the germline mutation falling after the 15RA is sufficient for tumour development. In contrast, when the germline mutation occurs before the 15RA, the proportion of LOH drops down to only a single case out of 65 tumours, showing that this type of germline mutation is not sufficient to allow the appearance of a tumour. Indeed, the second hit is a truncating mutation occurring after the 15RA in 97% of the cases. We concluded that colorectal cancer development tolerates APC truncation up to the end of the 15RA. This correlates almost perfectly with the ability of CtBP to stimulate APC oligomerization (Table 1).

CtBP and β-catenin compete for binding to the 15R of APC. The point mutations we have introduced in the 15R and that abolish CtBP binding are also those disrupting an interaction with β-catenin (35), suggesting that CtBP and β-catenin would compete for binding to the 15R. To test this, we performed immunoprecipitation experiments with an anti-flag antibody, using mixed extracts from HEK293T cells transiently expressing either fCtBP1, yAPC1247, yAPC1060, yAPC1020 or a YFP-β-catenin fusion construct (Fig. 10). We first confirmed that APC residues 1020–1060 were necessary for the interaction of fCtBP1 with truncated APC. However, the additional presence of YFP-β-catenin reduced and abolished the coimmunoprecipitation of yAPC1247 and yAPC1060 together with fCtBP1, respectively. We concluded that CtBP1 and β-catenin compete for binding to APC. Note, that the affinity of β-catenin for the 15RA is higher than for the other 15Rs (34), explaining the better competition of β-catenin for binding to yAPC1060 containing only the 15RA, than yAPC1247 containing all four 15Rs. In the case of yAPC1247, YFP-β-catenin was also detected in the immunoprecipitate, indicating that fCtBP1 and YFP-β-catenin can

**Figure 8.** APC dot formation requires dimerization of CtBP2. SW480 cells were transiently transfected with expression constructs for flag-CtBP1, flag-CtBP2, the dimerization defective mutant flag-CtBP2RR or the YFP-APC1485 fusion, either alone or in the indicated combinations. Thirty-six hours post-transfection, cells were stained with an anti-flag antibody and nuclei were revealed with the Hoechst dye.

**Figure 9.** The selection of APC truncating mutations in colorectal tumours from FAP patients correlates with the presence of a CtBP binding site in truncated APC. The diagram shows the distribution of the positions of somatic mutations in the APC protein sequence as a function of the position of the germline mutation. The analysis includes only tumours in which APC truncating mutations occur before the CID domain (before position 1417) and reported in Miyaki et al. (20), Albuquerque et al. (29), Crabtree et al. (30) and Lamlum et al. (31) (227 of 328 tumours). Among all tumours shown here, there was no mutation falling between positions 1020 and 1053. LOH, loss of heterozygocity. P < 10^-8 using the Fisher’s exact test for the comparison of tumour distribution in every germline category.
bind simultaneously to truncated APC when it contains all 15Rs.

DISCUSSION

We have described, in this study, the surprising property for the transcriptional repressor CtBP to promote oligomerization of truncated APC typical of colorectal cancer cells. This occurs through binding of CtBP to the 15Rs and requires also both dimerization domains of APC. Strikingly, the induction of APC oligomerization by CtBP correlates well with the minimal APC size resulting from the selection of truncating mutations found in tumours from FAP patients, indicating that it represents the minimal requirement for the successful development of a tumour.

APC oligomerization manifests itself as small round cytoplasmic inclusions so-called dots upon the ectopic expression of N-terminally tagged constructs, as described previously (35,53,54). Dots are unlikely the consequence of unspecific aggregation resulting from overexpression. First, the ectopic expression of truncated APC containing the CID results in a diffuse cytoplasmic pattern into a dotty nuclear one when endogenous APC is revealed using either the Ali (12–28) or the Ab-7 antibody, respectively. Together, these observations suggest that APC dots are probably physiological structures subjected to regulation.

The accompanying model illustrates how both CtBP1 and CtBP2 may induce and/or stimulate oligomerization of truncated APC (Fig. 11). The presented model is supported by the following observations. Spontaneous APC dot formation requires both domains bracketing the Armadillo repeat domain, each containing a dimerization domain. Stimulation by CtBP requires in addition binding of CtBP to either the 15RA, 15RC or 15RD which is influenced by the first dimerization domain. The lack of either dimerization domain is compensated, at least partially, by the presence of CtBP that is likely supporting each dimerization event. CtBP can bind to APC as a monomer, but only the dimer can promote dot formation. This model allows two predictions. First, APC dot stimulation requires CtBP dimerization, suggesting that stimuli influencing CtBP dimerization, i.e. phosphorylation by protein kinase A (57) or the concentration of nicotinamide adenine dinucleotide (56,58), would also impact on APC oligomerization. Second, CtBP and β-catenin compete for binding to the 15R of APC. HEK293T cells were transiently transfected with either a control vector (flag), an expression vector for flag-CtBP1, the indicated YFP-APC fusion constructs or an expression vector for β-catenin tagged at the N-terminus with YFP (yβcatenin). Thirty-six hours post-transfection, cell extracts were mixed as indicated and submitted to immunoprecipitation using an anti-flag antibody followed by western blotting using either the latter or an anti-GFP antibody. The positions of yβcatenin and the YFP-APC constructs are marked by an arrow-head and a bracket, respectively.
further development towards the emergence of a tumour. In a previous publication (27), we showed that APC constructs forming dots were able to inhibit the β-catenin degradation activity of longer APCs containing the CID and locating diffusely. It is tempting to hypothesize that CtBP-induced dots create a local environment with a large number of inhibitory binding sites for a component involved in β-catenin degradation, by analogy to what has already been described for Dvl (62). This inhibitory activity might be essential for tumour development, because the selective pressure imposes almost always the presence of a CtBP binding site in the truncated APC product resulting from mutation selection. The presence of the 15RA in at least one truncated APC product in tumours from FAP patients is sufficient, but it is still not optimal for tumour development. Accordingly, when a germline mutation falls before the 15RA, the selective pressure clearly leads to the retention of not only the 15Rs but also the first 20 amino acid repeat (20R1) in the APC product resulting from the somatic mutation (34). Our data indicate that the 20R1 influences neither CtBP binding nor dot formation, and it remains therefore to be determined why the 20R1 seems necessary for tumour development.

MATERIALS AND METHODS

Cells

Human embryonic kidney cells expressing the large T antigen from SV40 virus (HEK293T) and LoVo, HT29, SW480 and DLD1 colorectal cancer cell lines were all maintained in Dulbecco’s modified Eagle’s medium (PAA Laboratories, Co¨lbe, Germany) supplemented with 10% fetal calf serum (Perbio Laboratories, Frankfurt am Main, Germany) and 1% penicillin and 1% streptomycin (PAA Laboratories). APC mutations specific to each cell line are described in Rowan et al. (32).

Antibodies

The mouse anti-CtBP1 (E12) and goat anti-CtBP2 (E16) antibodies were purchased from Santa Cruz (Heidelberg, Germany). Secondary antibodies coupled to either horseradish peroxidase or Cy3 were from Dianova (Hamburg, Germany), the rabbit anti-flag from Sigma (Taufkirchen, Germany), the anti-GFP from Roche (Mannheim, Germany) and the anti-APC antibody Ali from Abcam (Cambridge, UK).

Plasmids

The plasmids expressing the YFP-APC fusion proteins were constructed by the standard molecular biology methods. The sequence of any of these plasmids is available upon request. The amino acid substitutions in the 15 amino acid repeats are described in Kohler et al. (35). pcDNAflag (15) was used as a control vector. Human N-terminally tagged flag-CtBP2 and flag-CtBP2RR (49) were obtained from G. Chinnaduray, human N-terminally tagged gfpCtBP1 (47) from M. Bienz and F. Hamada, human C-terminally tagged CtBP2gfp from J. Blaydes (55) and N-terminally tagged flag-APC1287 encoding mouse APC truncated at amino acid position 1287 from R. Fodde. Flag-CtBP1 was obtained by polymerase chain reaction (PCR) amplification with the Primers 5′-TAATTGCGGCCGCATGGCAGCTCGCACTG-3′ and 5′-TATCTGCGGCCGCCTACAACTGGTCACTG-3′, using the gfpCtBP1 plasmid as a template. The resulting PCR product was inserted into the NotI site of the pcDNA flag vector and verified by sequencing. The expression plasmid for the YFP-β-catenin fusion has been described previously (63).

Transfections

Plasmids were transfected into cells overnight using 5 μl of polyethyleneimine (1 mg/ml) per μg DNA. For transient transfection of plasmids in DLD1 or SW480 cells, 2 μg of total DNA/2 50 000 cells/35 mm dish was used. For transient transfection of plasmids in HEK293T cells, 10 μg of total DNA/50 000 cells/85 mm dish was used. Western blotting was performed according to Behrens et al. (15). The blots were developed using the chemiluminescence reagents Western Lightning™ (Perkin Elmer Life Sciences, Boston, MA, USA) and the signals were detected under a LAS-3000-Fuji camera from Raytest (Straubenhardt, Germany). Immunoprecipitation and immunofluorescence were conducted as described (15).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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