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Functional definition of the mutation cluster region of adenomatous polyposis coli in colorectal tumours

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The mutation cluster region (MCR) of adenomatous polyposis coli (APC) is located within the central part of the open reading frame, overlapping with the region encoding the 20 amino acid repeats (20R) that are β-catenin-binding sites. Each mutation in the MCR leads to the synthesis of a truncated APC product expressed in a colorectal tumour. The MCR extends from the 3′ border of the first 20R coding region to approximately the middle of the third 20R coding region, reflecting both positive and negative selections of the N- and C-terminal halves of the APC protein in colon cancer cells, respectively. In contrast, the second 20R escapes selection and can be either included or excluded from the truncated APC products found in colon cancer cells. To specify the functional outcome of the selection of the mutations, we investigated the β-catenin binding capacity of the first three 20R in N-terminal APC fragments. We found in co-immunoprecipitation and intracellular co-localization experiments that the second 20R is lacking any β-catenin binding activity. Similarly, we also show that the tumour-associated truncations abolish the interaction of β-catenin with the third 20R. Thus, our data provide a functional definition of the MCR: the APC fragments typical of colon cancer are selected for the presence of a single functional 20R, the first one, and are therefore equivalent relative to β-catenin binding.

INTRODUCTION

Inactivation of the tumour suppressor adenomatous polyposis coli (APC) is the earliest known mutagenic event that initiates tumoral progression towards the development of colon cancer (1). APC is a crucial component of the so-called β-catenin destruction complex that catalyses the efficient phosphorylation of β-catenin, which is subsequently degraded in the proteasome (2). In colon cancer, one consequence of APC inactivation is the stabilization of β-catenin that in turn feeds the cell with a permanent mitogenic signal (3,4). Indeed, a plethora of data has proven that the stabilization of β-catenin is an essential driving force allowing the hyperproliferation of the epithelial cells of the colon (5–8).

The central region of the 2843 amino acid APC sequence (9) harbours interaction domains involved in binding and/or regulation of β-catenin. They include the so-called 15 amino acid repeats (15R, labelled A–D) (10) and 20 amino acid repeats (20R, numbered 1–7) that are β-catenin binding sites and the SAMP repeats that interact with axin/conductin (11) (Fig. 1). Thus, APC can bring β-catenin in the vicinity of axin/conductin that in turn recruits casein kinase I and glycogen synthase kinase 3β. These kinases catalyse the sequential phosphorylation of β-catenin, which is an essential prerequisite for proteasomal degradation (12–15).

Both alleles of APC are mutated in the vast majority of colon tumours (16–21). The mutations are either nonsense substitutions or frame shifts resulting from small insertions or deletions that affect the open reading frame. Interestingly, several mutational hotspots occur within direct repeated sequences, likely the consequence of replication errors (22). Allelic loss, which implies deletion of the whole open reading frame or somatic conversion (23) also contributes to APC inactivation. As a consequence of these mutational events, colon cancer cells express only truncated versions of APC (21).

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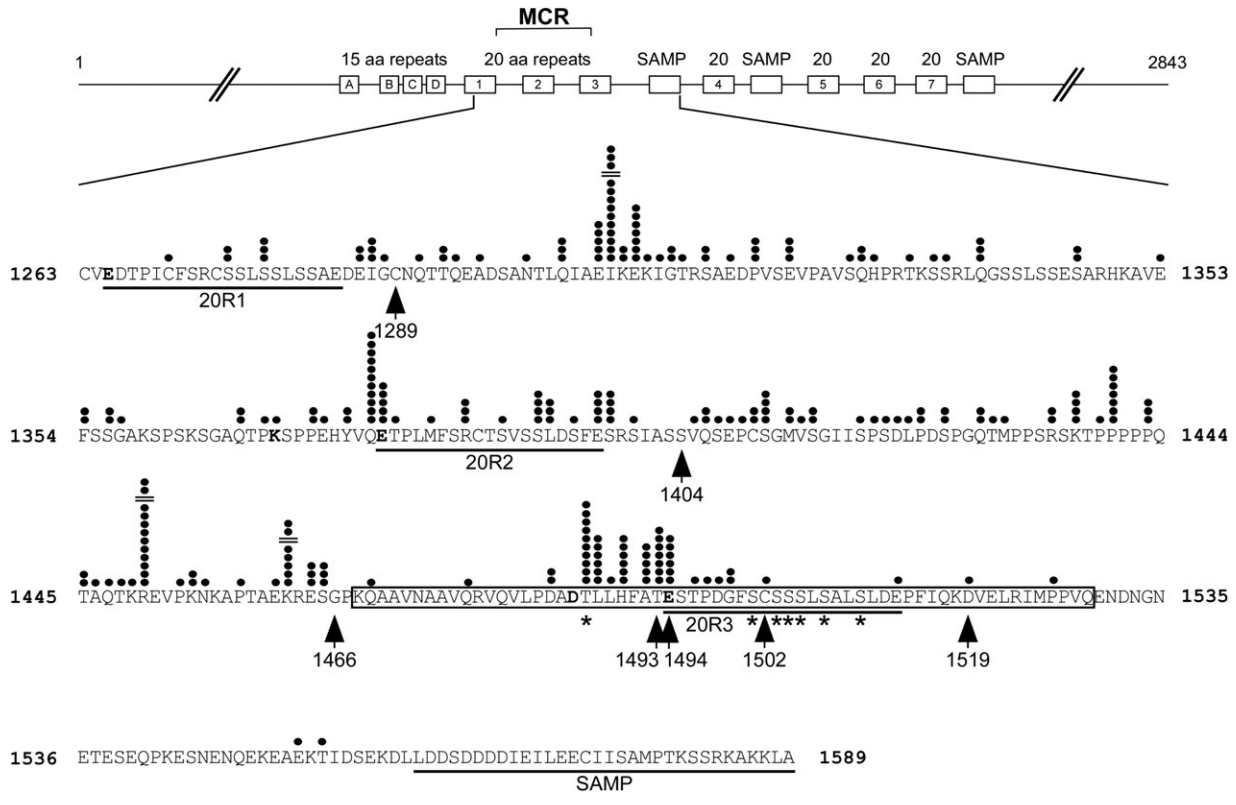

Figure 1. Schematic representation of APC and amino acid sequence of the MCR and flanking regions. The axin/conductin binding sites (SAMP) and the 15 and 20R as well as the position of the MCR are indicated above the scheme. Each dot above the sequence is representative of a nonsense or frame-shift somatic mutation found in a colorectal tumour that led to the synthesis of a truncated APC product. The double bars indicate 10 more mutations. The 20R are underlined and the interaction interface of the 20R3 with β-catenin (31,32) is boxed. The charged residues that are making crucial contacts with β-catenin are marked with bold letters. Known phosphorylation sites in the 20R3 are marked with asterisks. Amino acid positions are indicated with arrows. The scheme is modified from (43).

In the familial adenomatous polyposis coli (FAP) syndrome, one mutated APC allele is transmitted through the germ-line (24). Subsequent somatic mutations inactivate the second allele and precede the development of many polyps early in the adulthood that evolve systematically into malignant tumours. This hereditary disease allowed observing that the sequence of mutational events affecting both alleles is not a random process (25–27). If the germ-line defect is either a loss of heterozygosity or a truncating mutation occurring before the first 20R, then a truncating mutation will affect the remaining allele after the first 20R. Thus, there is a strong selection for the retention of at least one truncated APC product containing the first 20R. The importance of this selection has been highlighted in a recent report showing that truncated APC is required for optimal cell proliferation (28). In addition, there is also an almost systematic selection for truncating mutations occurring before the first SAMP repeat. Thus, it is thought that the truncating mutations are selected to preclude the formation of a functional destruction complex by eliminating the binding sites for axin/conductin (29).

The consequence of the double—positive and negative—selection is that the recorded mutations from FAP patients accumulate between the first 20R and the first SAMP repeats, in the so-called mutation cluster region (MCR) (19–21) (Fig. 1). With only very few exceptions, the 5′ border of the MCR is clearly located just after the first 20R and we have shown recently that it is an important structural motif allowing truncated APC to keep some residual control on β-catenin activity (30). The 3′ border of the MCR is less well defined. Although the selected truncated APC isoforms almost systematically lack the first SAMP repeat, there is an apparent mutation gap between this motif and position 1494 where a bulk of mutations accumulate, suggesting that amino acid 1494 represents a critical position beyond which only few mutations are permissive for tumour development. Interestingly, the structure of the third 20R in a complex with β-catenin (31,32) reveals two parts that provide important contact sites to β-catenin. The C-terminal portion after position 1494 is characterized by a cluster of serines that drastically enhance the affinity for β-catenin when they are phosphorylated. The N-terminal portion contains D1486 and E1494 that are crucial residues directly interacting with K435 and K312, the two so-called charged buttons in the β-catenin armadillo repeat. These two lysine residues were also observed as direct contact points in the very similar structures of β-catenin complexed with either E-cadherin (33) or Tcf3 (T-cell factor) (34) and their individual mutation into aspartate has been shown to abolish the interaction of β-catenin with Tcf3 (34). These data suggested that APC truncations might be selected for altered β-catenin binding activity to the third 20R.

Similarly, it is striking that the second amino acid repeat is apparently left outside any selective pressure. Interestingly, the first negatively charged amino acid residue that is interacting directly with one of the two charged buttons of β-catenin in the other 20R is exchanged for a lysine at position 1370 in the second 20R (31,32). In addition, it has been established recently that among all APC-derived repeats displaying a β-catenin binding activity, the second 20R had the lowest affinity when they were analysed individually *in vitro* (35). These observations suggested that the second 20R might be unable to bind β-catenin in the context of a truncated APC.

In this study we investigated the ability of truncated APC to bind to β-catenin. Our data reveal that only the first 20R is functional for β-catenin binding, whereas the second repeat and the remaining half of the third repeat up to position 1493 cannot bind to β-catenin. Thus, our data provide a rationale for the absence of selection of the second 20R and for the abrupt drop of APC mutation frequency after position 1494 in the middle of the third 20R.

RESULTS

To investigate the ability of the 20R2 (second 20R) to bind to β-catenin in the context of a truncated APC molecule, we first compared the activities of YFP(yellow fluorescent protein)-APC fusions containing (yAPC1404) or lacking (yAPC1289) the 20R2 (Figs 1 and 2). Co-immunoprecipitation experiments indicated that both of them were binding to β-catenin with a very similar efficiency. Thus, the presence or absence of the 20R2 does not modify β-catenin binding. As the binding of β-catenin to the 15R and 20R1 (first 20R) may be too strong to observe the contribution of the 20R2, we disrupted the β-catenin binding activity of the four 15R and the 20R1 by exchanging residues crucial for the interaction. In the crystallographic structure of β-catenin in a complex with the first 15R (36), Y1027 and Y1031 of APC make direct contacts with R386 of β-catenin. Mutation of the latter residue into alanine drastically affected the association with the first 15R (37). Therefore Y1027 and Y1031 were exchanged for alanine or leucine whereas the highly conserved S1028 for glycine (Fig. 3A). The consequence of these mutations was investigated in a hybrid protein made of YFP fused to the first mutated 15R [yAPC(959–1037)15Rμ] whose β-catenin-binding activity was compared with that of the corresponding wild-type construct [yAPC(959–1037)]. Immunoprecipitation experiments revealed that the three residue replacements were sufficient to abolish the interaction of β-catenin with the first 15R (Fig. 3B).

Similarly, to hamper β-catenin binding to the 20R1, we exploited its property to bind to β-catenin with a high affinity only when it is phosphorylated (31,32,35,38,39). Therefore, S1272, S1275, S1276, S1278 and S1281 were mutated into alanine in the context of an APC sequence extending from amino acid 2 to 1289 fused to YFP (yAPC1289-20R1μ) (Fig. 3C). The β-catenin binding activity of this construct, the corresponding wild-type fusion (yAPC1289) and shorter constructs lacking either 20R1 (yAPC1247) or both all 15R and the 20R1 (yAPC891) were compared. Immunoprecipitation experiments revealed that among the four fusion proteins

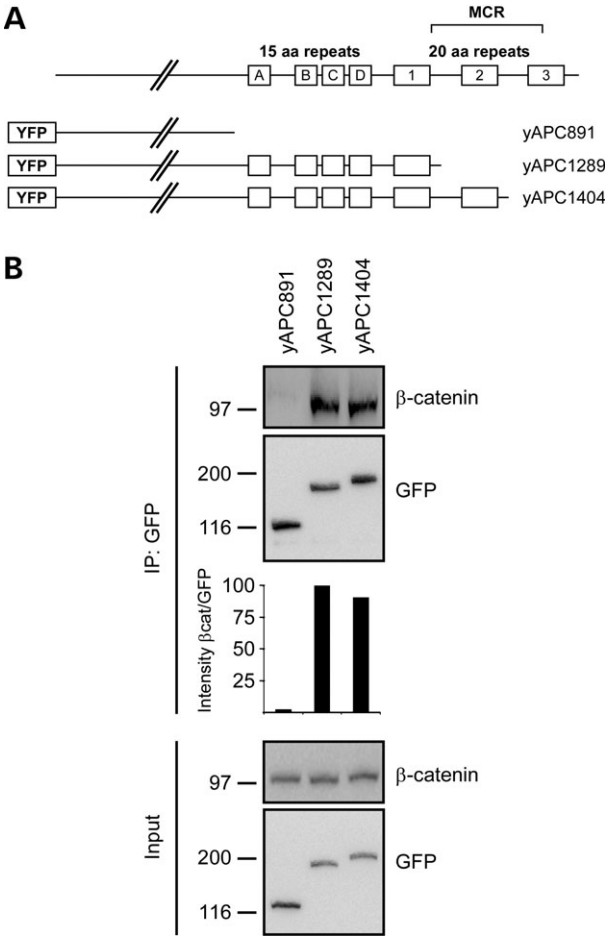

Figure 2. Deletion of the second 20R (20R2) of APC does not affect the interaction with endogenous β-catenin. (**A**) Schematic representation of YFP-APC constructs. (**B**) Transient transfection of HEK293T cells with constructs indicated above the lanes was followed by immunoprecipitation with an anti-GFP antibody and western blotting using either anti-GFP or anti-β-catenin antibody. A densitometry analysis reports the β-catenin/yAPC ratio of the signals seen on the blots.

only yAPC1289 was displaying a strong β-catenin binding activity (Fig. 3D). In contrast, yAPC891 showed no binding to β-catenin, whereas yAPC1247 and yAPC1289-20R1μ were binding only weakly owing to the presence of the 15R. Thus, the mutations introduced in the 20R1 were efficient in abolishing its interaction with β-catenin.

To characterize the β-catenin binding activity of the 20R2, the mutations described above were introduced in all four 15R and the 20R1 in the context of a truncated APC extending from amino acid 2 to 1404 and therefore containing an intact 20R2 (yAPC1404-15Rμ-20R1μ) (Fig. 4A). As negative controls, we used yAPC891 as well as yAPC1289 where all the four 15R and the 20R1 were mutated (yAPC1289-15Rμ-20R1μ). A positive control consisted of yAPC1289 containing four mutated 15R but a wild-type 20R1 (yAPC1289-15Rμ). Immunoprecipitation experiments revealed that yAPC1289-15Rμ was binding efficiently to β-catenin relative to yAPC891 (Fig. 4B). In contrast, yAPC1289-15Rμ-20R1μ displayed only a background activity similar to yAPC891, confirming that the mutations

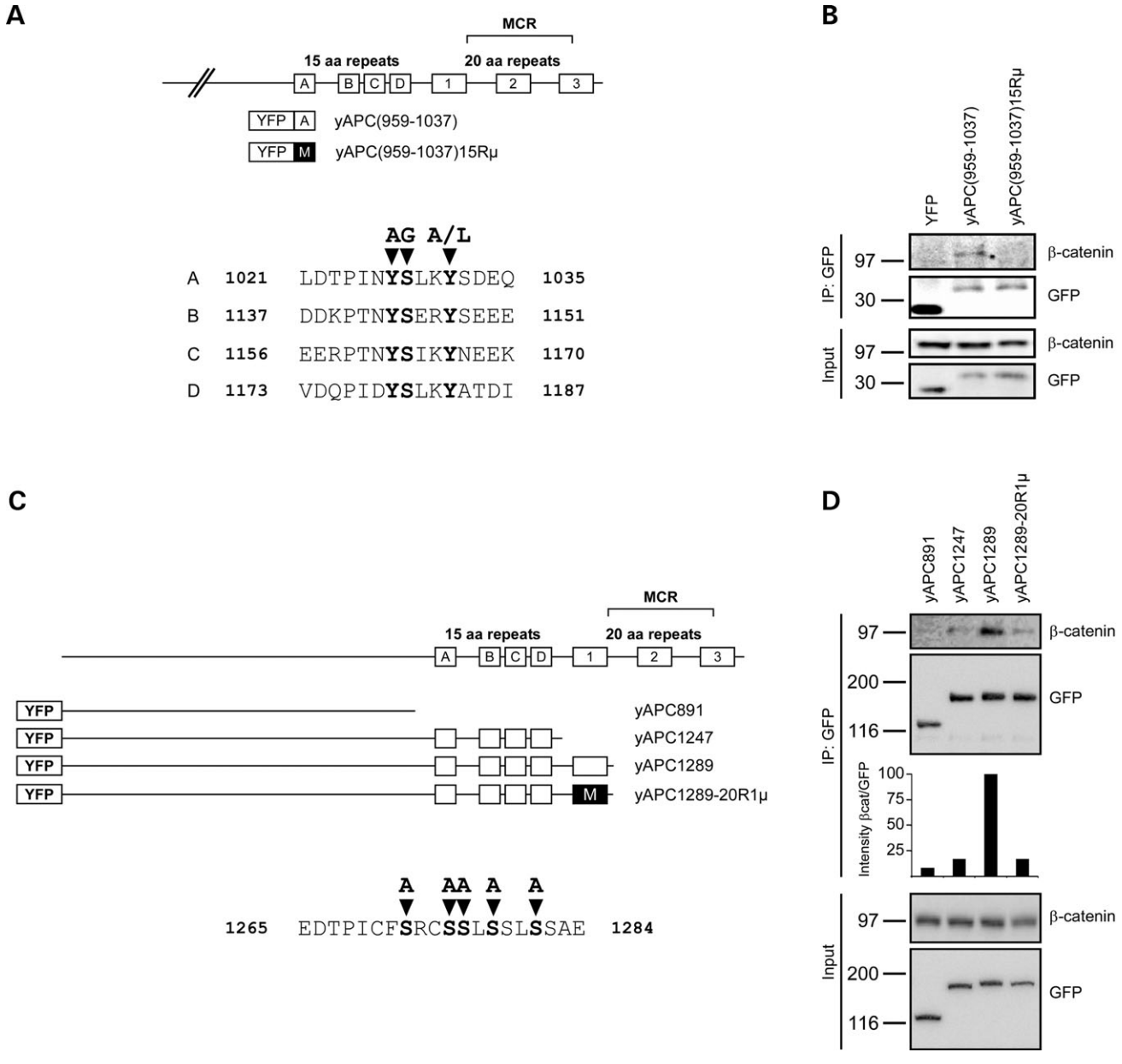

Figure 3. Mutations in the 15 and 20R of APC affect the interaction with endogenous β-catenin. (**A**) The sequences of the 15R (A–D) are shown together with their positions in the full length APC molecule. The amino acid residues highlighted in bold were replaced by alanine or glycine in all the four 15R, except by leucine for the tyrosine at the third position in 15R-B. The YFP-APC fusion proteins used in B are shown schematically. An M-labelled black box represents a mutated repeat. (**B**) The effect of the mutations described in A on β-catenin binding is shown for the first 15R only. To test this, transient transfection of SW480 cells with the indicated constructs was followed by immunoprecipitation with an anti-GFP antibody and western blotting using either anti-GFP or anti-β-catenin antibody. (**C**) Schematic representation of the YFP-proteins used in D and amino acid sequence of the 20R1. The serines indicated with arrows were replaced by alanine in the yAPC1289-20R1μ construct. An M-labelled black box represents a 20R1 containing the mutations. (**D**) Transient transfection of HEK293T cells with the constructs shown in C was followed by immunoprecipitation with an anti-GFP antibody and western blotting using either anti-GFP or anti-β-catenin antibody. A densitometry analysis reports the β-catenin/yAPC ratio of the signals seen on the blots.

introduced in the repeats were efficiently reducing the association with β-catenin. Moreover, extending yAPC1289-15Rμ-20R1μ with the 20R2 as seen in yAPC1404-15Rμ-20R1μ did not restore any β-catenin binding activity, indicating that the 20R2 cannot interact with β-catenin in these settings.

To confirm this result, we measured the transcriptional activity of β-catenin in a reporter assay. As positive controls, we used expression vectors encoding either a full length YFP-APC fusion or a shorter version extending up to the end of the first SAMP repeat (yAPC1641). These two constructs efficiently inhibited the transcriptional activity of β-catenin (Fig. 4C). yAPC1289 and yAPC1404 were also inhibiting the transcriptional activity of β-catenin, with a similar efficiency. This inhibition required binding to β-catenin, because yAPC1289-15Rμ-20R1μ was inactive in the assay.

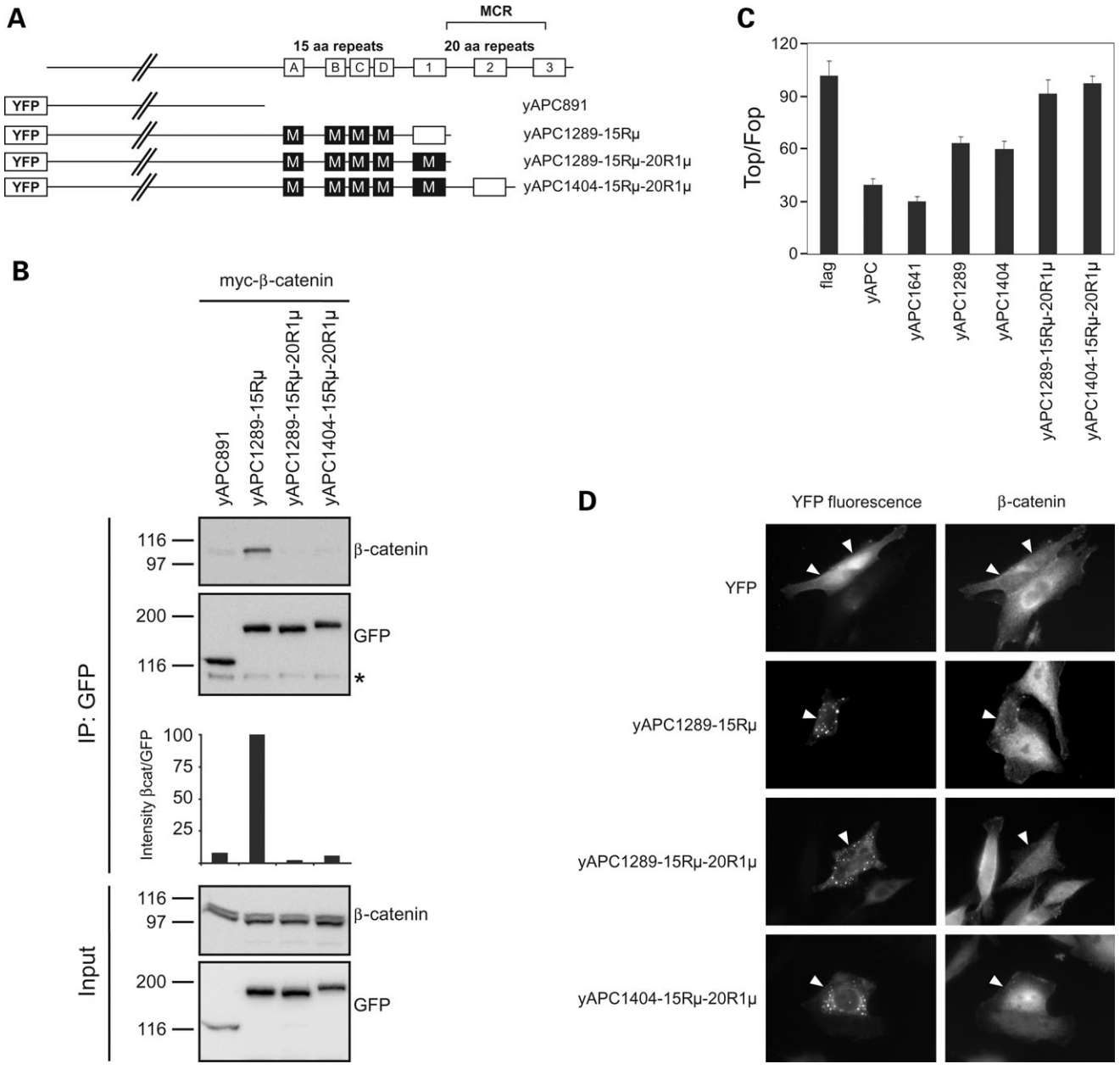

Figure 4. The second 20R (20R2) of APC does not bind to β-catenin. (**A**) Schematic representation of YFP-APC constructs. An M-labelled black box represents a repeat mutated as shown in Figure 3. (**B**) Transient transfection of HEK293T cells with the indicated YFP-APC fusions constructs and a myc-β-catenin expression plasmid was followed by immunoprecipitation with an anti-GFP antibody and western blotting using either anti-GFP or anti-β-catenin antibody. The asterisk indicates an unspecific band. A densitometry analysis reports the β-catenin/yAPC ratio of the signals seen on the blots. (**C**) SW480 cells were transiently transfected with either a β-catenin-dependent reporter gene (TOP) or a control reporter gene (FOP) and either an empty vector (flag) or the indicated expression vectors. Represented is the mean TOP/FOP ratio of three experiments. FOP activities were not significantly altered in the different conditions. (**D**) Transient transfection of SW480 cells with the indicated constructs was followed by immunostaining using an anti-β-catenin antibody. Arrowheads indicate transfected cells.

Addition of the 20R2, as seen in yAPC1404-15Rμ-20R1μ, did not restore the inhibiting activity. Thus, this experiment suggested that the 20R2 does not contribute to the down-regulation of β-catenin-dependent transcription.

To investigate the ability of 20R2 to associate with β-catenin inside the cells, we used the property of truncated APC to build intracellular inclusions (40). We also observed previously (30) that β-catenin co-localizes in these structures and we hypothesized that this was dependent on the ability of APC to interact with β-catenin. Therefore, YFP, yAPC1289-15Rμ, yAPC1289-15Rμ-20R1μ and yAPC1404-15Rμ-20R1μ were transiently expressed in SW480 cells where endogenous β-catenin can be easily visualized by immunofluorescence staining (Fig. 4D). In this experiment YFP localized diffusely in both the nucleus and the cytoplasm, whereas the three yAPC fusion proteins formed in addition

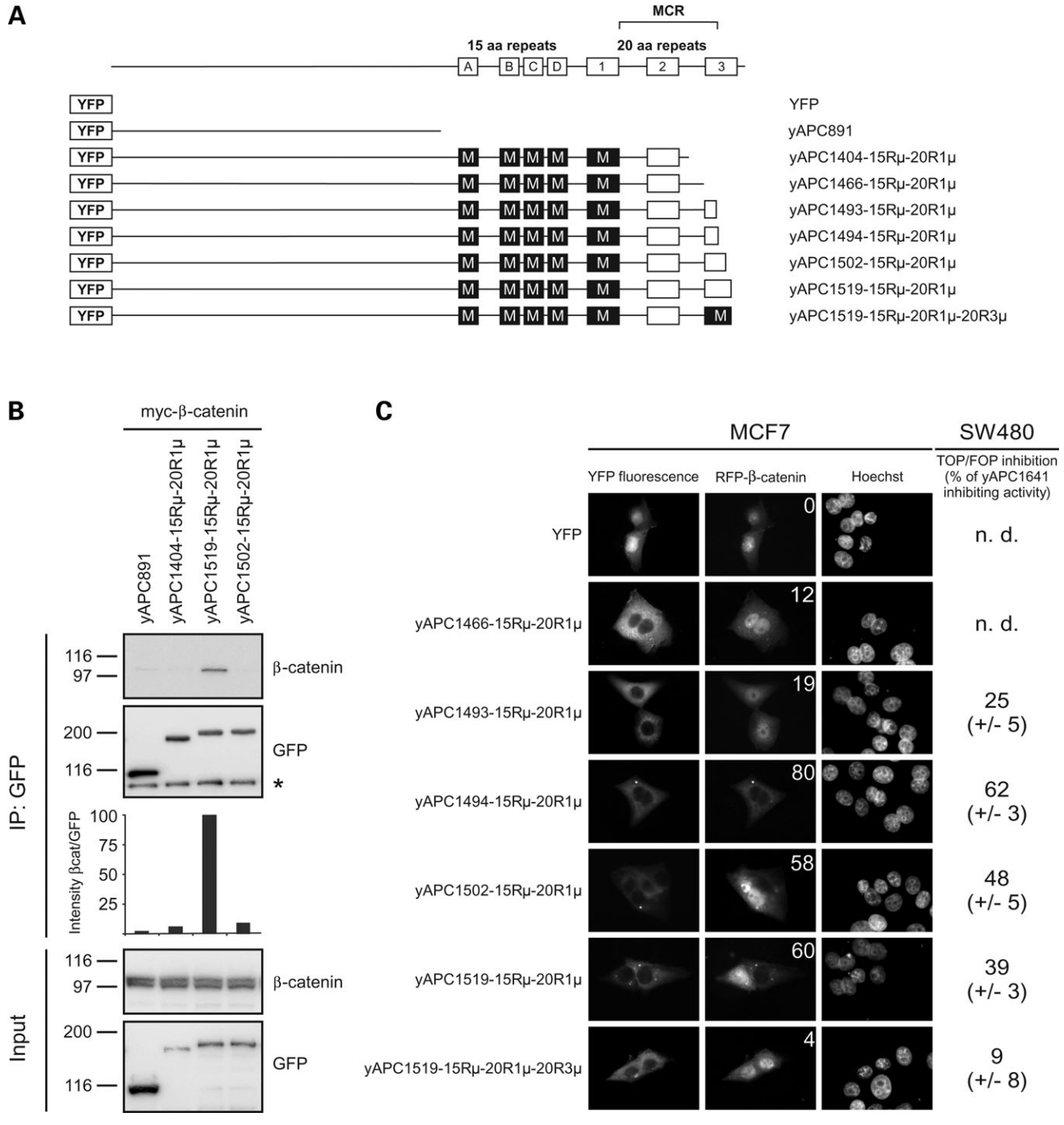

Figure 5. β-Catenin binding to the 20R3 defines the 3′ border of the MCR. (**A**) Schematic representation of YFP-APC constructs. An M-labelled black box represents a mutated repeat. (**B**) Transient transfection of HEK293T cells with the indicated YFP-APC fusion constructs and a myc-β-catenin expression plasmid was followed by immunoprecipitation with an anti-GFP antibody and western blotting using either anti-GFP or anti-β-catenin antibody. The asterisk indicates an unspecific band. A densitometry analysis reports the β-catenin/yAPC ratio of the signals seen on the blots. (**C**) RFP-β-catenin recruitment in MCF7 cells and TOP/FOP reporter assays in SW480 cells. Transient transfection of MCF7 cells with the indicated constructs together with an RFP-β-catenin expression plasmid was followed by immunofluorescence analysis. The numbers indicate the percentage of RFP-β-catenin-positive cells ($n = 100$) displaying only cytoplasmic RFP-β-catenin. Hoechst, DNA staining. TOP/FOP reporters ratios were determined as described in legend of Figure 4C after transfection of yAPC1641 and the indicated constructs in SW480 cells. Inhibition of the TOP/FOP ratio is given relative to the inhibiting activity of yAPC1641, which was set to 100%.

cytoplasmic inclusions. β-catenin partially co-localized in these inclusions when yAPC1289-15Rμ was expressed. In contrast, the β-catenin staining pattern remained diffuse in the presence of yAPC1289-15Rμ-20R1μ, thus revealing the efficiency of the mutations introduced in the four 15R and the 20R1 in destroying the interaction with β-catenin.

As the β-catenin staining remained also diffuse in cells expressing yAPC1404-15Rμ-20R1μ, we concluded that the 20R2 cannot bind to β-catenin in this experiment.

To investigate the capacity of the 20R3 (third 20R) to interact with β-catenin, the construct yAPC1404-15Rμ-20R1μ was extended up to either approximately the middle of the 20R3 at position 1502 (yAPC1502-15Rμ-20R1μ) or shortly after at position 1519 (yAPC1519-15Rμ-20R1μ) (Figs 1 and 5A). Immunoprecipitation experiments confirmed the lack of interaction between yAPC1404-15Rμ-20R1μ and β-catenin (Fig. 5B). yAPC1519-15Rμ-20R1μ was binding strongly to β-catenin, but yAPC1502-15Rμ-20R1μ did not display any β-catenin binding activity. Thus, residues 1502–1519 represent an important determinant of the interaction. This is in line with the role of the serine residues located in this portion and whose phosphorylation has been shown to drastically enhance the affinity of the 20R3 for β-catenin (31,32,38,41).

To visualize the interaction of β-catenin with the 20R3 inside the cells, we employed MCF7 cells in which truncated APC constructs located in the cytoplasm and depleted the nucleus from co-expressed RFP(red fluorescent protein)-β-catenin (Fig. 5C). For this experiment, several constructs were designed, all of them containing mutated 15R and 20R1 and extending from amino acid 2 to either 1494 (yAPC1494-15Rμ-20R1μ), 1493 (yAPC1493-15Rμ-20R1μ) or 1466 (yAPC1466-15Rμ-20R1μ) (Fig. 5A). In addition, we also created yAPC1519-15Rμ-20R1μ-20R3μ as a negative control where five known phosphorylatable serine residues in the 20R3 (S1501, S1504, S1505, S1507 and S1510) (31) were mutated into alanine and where E1494 and D1486 (Fig. 1) that are making direct contacts with β-catenin (31,32) were also exchanged for alanine residues. E1494 and D1486 were mutated in addition to the serines to destroy efficiently any residual β-catenin binding activity, because an unphosphorylated 20R3 displays an affinity for β-catenin in the same range as a phosphorylated 20R1 *in vitro* (35). These constructs, as well as yAPC1519-15Rμ-20R1μ, yAPC1502-15Rμ-20R1μ and YFP were transiently expressed in MCF7 cells together with a RFP-β-catenin fusion protein. Immunofluorescence observation showed that YFP and RFP-β-catenin localized diffusely throughout the cell when they were co-expressed, mainly in the nucleus (Fig. 5C). All yAPC constructs were located predominantly, if not exclusively, in the cytoplasm and there was no apparent difference of fluorescence intensity between them. In the presence of either yAPC1519-15Rμ-20R1μ or yAPC1502-15Rμ-20R1μ, RFP-β-catenin was absent from the nucleus of 60 and 58% of RFP-β-catenin-positive cells, respectively. This effect was concentration-dependent because β-catenin was found in the nucleus of cells expressing either high amounts of RFP-β-catenin or low amounts of the yAPC fusions. In contrast, RFP-β-catenin was located predominantly in the cytoplasm of only 4 and 12% of the cells expressing either yAPC1519-15Rμ-20R1μ-20R3μ or yAPC1466-15Rμ-20R1μ. Thus, as described earlier (42), a retention mechanism prevents β-catenin from reaching the nucleus and this occurs through binding to the 20R3 because the retention effect is abolished when the essential features responsible for a high β-catenin binding affinity are mutated in the 20R3 or when the entire repeat is deleted. yAPC1494-15Rμ-20R1μ had the best retention capacity, since RFP-β-catenin was almost exclusively cytoplasmic in 80% of positive cells. Remarkably, shortening yAPC1494-15Rμ-20R1μ by the removal of a single amino acid residue (yAPC1493-15Rμ-20R1μ) resulted in a drastic impairment of the retention, since RFP-β-catenin was predominantly cytoplasmic in only 19% of cells expressing yAPC1493-15Rμ-20R1μ, near the level observed with yAPC1466-15Rμ-20R1μ where the entire repeat has been deleted. A western blotting analysis indicated that differential expression could not explain these differences (unpublished data). Thus amino acid 1494 represents a crucial limit below which β-catenin recruitment is barely detectable. To confirm this result, the different abilities of the yAPC constructs to inhibit the transcriptional activity of β-catenin were measured in the TOP/FOP reporter assay (Fig. 5C). We found a good correlation between the ability of the constructs to recruit β-catenin and to inhibit its transcriptional activity, highlighting again the importance of amino acid 1494. Strikingly, a bulk of natural APC truncating mutations is selected up to position 1494 (Fig. 1), after which the frequency of mutations drops drastically. These observations strongly suggest that APC mutations are selected for altered β-catenin binding to the 20R3.

Several natural mutations however occur after the critical 1494 position (Fig. 1). This situation is represented by our two constructs extending up to positions 1502 or 1519. Interestingly, yAPC1519-15Rμ-20R1μ and yAPC1502-15Rμ-20R1μ display a reduced ability to retain RFP-β-catenin in the cytoplasm when compared with yAPC1494-15Rμ-20R1μ (Fig. 5C). This suggests that β-catenin binding might also be altered despite the presence of the phosphorylatable residues in some rare cases. The natural frame-shift mutations occurring in codons 1502 and 1519 result in the addition of six extra amino acids after position 1502 and the replacement of D1519 by a glutamate residue, followed by a termination codon (21). These sequence alterations were incorporated in our constructs but they had no apparent influence on RFP-β-catenin recruitment in comparison with yAPC1519-15Rμ-20R1μ and yAPC1502-15Rμ-20R1μ (unpublished data). Thus, our data suggest that mutations occurring after position 1494 are exceptions that are selected because of altered β-catenin binding activity to the 20R3.

DISCUSSION

Almost all APC mutations that are selected in colorectal tumours lead to the synthesis of truncated APC products that contain a 20R1 fully competent in β-catenin binding. In contrast, the mutations distribute on either sides of the 20R2. To understand why there is no apparent selective pressure for the presence or the absence of the 20R2, we analysed its interaction with β-catenin in the context of a truncated APC molecule containing inactivated 15R and 20R1. We found that the 20R2 cannot bind to β-catenin. Similarly, we also revealed that the remaining piece of the 20R3 that is retained in many truncated APC molecules is unable to interact efficiently with β-catenin, indicating that mutations are selected for altered β-catenin binding to the 20R3. Thus, our results

strongly suggest that the conditions for a successful selection of an APC mutation rely on keeping one single functional 20R, the 20R1, and therefore provide a functional definition of the MCR in colorectal tumours.

The lack of β-catenin binding activity of the 20R2 was investigated not only in co-immunoprecipitation experiments in HEK293T (human embryonic kidney 293T) cells, but also directly inside the cells in co-localization experiments in SW480 cells. Despite their high expression levels relative to endogenous APC in SW480 cells (unpublished data), β-catenin was always localizing independently of the exogenous APC constructs containing mutated 15R and 20R1 but wild-type 20R2. Thus our data provide the *in vivo* confirmation that the 20R2 cannot bind to β-catenin. They complement the previous *in vitro* observations that highlighted the very weak affinity of the 20R2 towards β-catenin when it was analysed individually using isothermal titration calorimetry, outside the APC context (35). They also provide an explanation for the presence of a lysine residue at position 1370 in the 20R2 instead of an acidic residue directly interacting with β-catenin in the 20R1 and the 20R3 (31,32,34).

The β-catenin binding activity of the 20R3 was first investigated in co-immunoprecipitation experiments in HEK293T cell extracts. A construct extending up to position 1519 in a 15R- and 20R1-mutated background (yAPC1519-15Rμ-20R1μ) was binding efficiently to β-catenin. In contrast, the deletion of residues 1503–1519 from this construct (as in yAPC1502-15Rμ-20R1μ) abolished the interaction with β-catenin (Fig. 5B). This result was obtained in an immunoprecipitation experiment where the analysed components are diluted upon addition of the cell-lysis buffer and immunoprecipitates extensively washed. This drives the equilibrium of the interaction towards the dissociation. In contrast, in co-localization experiments in MCF7 cells, the construct yAPC1502-15Rμ-20R1μ was retaining β-catenin in the cytoplasm. We interpret this observation as the consequence of the interaction of RFP-β-catenin with the remaining N-terminal half of the 20R3. The C-terminal half of the 20R3 contains several serine residues at positions (S1501, S1503, S1504, S1505, S1507 and S1510) and their phosphorylation enhances the affinity of the repeat for β-catenin (31,32). However, an *in vitro* analysis performed with individual repeats revealed that even in an unphosphorylated state, 20R3 displays an affinity towards β-catenin comparable with a phosphorylated 20R1 (35). Our data indicated indeed that a 20R3 lacking five known phosphorylatable serine residues (yAPC1502-15Rμ-20R1μ) could still efficiently recruit β-catenin inside the cells under the conditions of ectopic expression, likely because the concentrations of the components were sufficiently high to compensate for the decrease of affinity resulting from the deletion of the C-terminal half of the 20R3.

We pursued our deletion analysis to establish the limit under which β-catenin recruitment would not be detectable anymore. We found that the removal of E1494 (yAPC1493-15Rμ-20R1μ) acted almost as a digital switch, sufficient to affect seriously the recruitment of RFP-β-catenin, whereas the construct containing this residue (yAPC1494-15Rμ-20R1μ) was fully competent to interact with β-catenin. The crystallographic structure of a 20R3 in a complex with β-catenin (31,32) indicates that D1486 and E1494 are two negatively charged residues that form salt bridges with the two so-called positively charged buttons of β-catenin in a crucial manner (31,32,34). Thus, under conditions of ectopic expression, a strong reduction of β-catenin recruitment to the 20R3 requires the removal of E1494 in addition to the deletion of the phosphorylatable residues. The residual activity could be abolished by further deletion of the remaining N-terminal half of the 20R3, which is also known to interact with β-catenin, particularly the region located between the two important negatively charged residues (31,32). Strikingly, the E1494 limit coincides with the position where many recorded natural mutations accumulate (Fig. 1). A few positions occurring after the critical E1494 position are however tolerated during tumour progression. We showed that the 20R3 as found in the context of molecules extending up to positions 1502 or 1519 and reproducing the situations in tumour samples (21) recruit β-catenin less efficiently than a shorter construct extending up to amino acid 1494 despite conditions of over-expression. This suggests that the few truncated APC that are selected following a mutational hit occurring after position 1494 display a 20R3 with an altered β-catenin-binding activity, even if the phosphorylatable residues are present as in yAPC1519-15Rμ-20R1μ. This situation is however rarely observed, likely because β-catenin binding is less affected in comparison with truncations occurring before position 1494. An effective selection might require in addition the reduction of the APC level, as we observed it in HT29 cells containing a truncating mutation at position 1555 and expressing lower amounts of APC than SW480, DLD1 and LoVo colon cancer cells where APC is truncated before position 1494 (unpublished data). Alternatively, other proteins affecting the interaction with the third repeat may contribute to the selection.

A nuclear export signal (NES) located within the phosphorylatable region of the 20R3 between positions 1505 and 1512 has been previously described (43). According to this work, APC truncations would be selected for the lack of this NES, implying an altered ability to export β-catenin from the nucleus. Whatever the outcome of the current discussion about the nuclear localization of APC is (44), there is still a gap between the core sequence of the putative NES and the accumulation of mutations at position 1494, suggesting that an additional factor is involved in the selection. Our data strongly support the notion that truncated APC are selected for impaired β-catenin binding to the 20R3, in addition to the removal of the axin/conductin binding sites and the NES. We propose that E1494 represents the 3′ border of the MCR and that mutations selected after this position are exceptions whose phenotype is expressed in an analogous way.

Truncated APC molecules selected in colon cancer retain almost systematically a functional 20R1, although some rare exceptions must be also highlighted (Fig. 1). As we have shown here that the 20R2 cannot bind to β-catenin and that the 20R3 is inactivated, this drives to the conclusion that truncated APC are selected for the presence of a single functional 20R. The selection itself shows that some β-catenin binding activity must be kept and that it is crucial for the tumour cell. Actually, the presence of truncated APC seems essential for optimal cell proliferation (28). At the molecular level, the reason for the selection is not yet clear. Several studies have

highlighted the potential of truncated APC to downregulate the transcriptional activity of β-catenin (45–47) even without affecting its stability (30), but the consequences in terms of intracellular signalling remain unravelled. Nevertheless, the present study strongly suggests that truncated APC are selected for one common feature, an equivalent ability to interact with β-catenin. It provides a functional definition of the MCR.

MATERIALS AND METHODS

Materials

ProtA/G-sepharose was from Santa Cruz Biotechnologies (Heidelberg, Germany) and Hoechst 33258 from Sigma (Steinheim, Germany).

Cells

HEK293T, MCF7 and SW480 colon cancer cells were maintained in DMEM medium (PAA Laboratories, Cölbe, Germany) supplemented with 10% fetal calf serum (Perbio Laboratories, Frankfurt am Main, Germany) and 1% penicillin and 1% streptomycin (PAA Laboratories).

Antibodies

Rabbit antibody H102 against β-catenin was purchased from Santa Cruz. Secondary antibodies coupled to either horseradish peroxidase or Cy3 were from Dianova (Hamburg, Germany), anti-β-actin from Santa Cruz Biotechnologies (Heidelberg, Germany), and anti-GFP (green fluorescent protein) from Roche (Mannheim, Germany).

Plasmids

Plasmids expressing the YFP-APC fusion proteins were constructed using standard molecular biology methods, using pCMV-APC2843 (48) as a template for PCR (polymerase chain reaction) reactions and YFP-APC (42) as a recipient vector. The plasmid RFP-β-catenin was created using mRFP (49) as a template for PCR and YFP-β-catenin (42) as the recipient vector. The sequence of any of these plasmids is available upon request. The plasmid expressing myc-β-catenin was described earlier (50).

Transfection

Plasmids were transfected into cells overnight using 5 μl polyethylenimine (1 mg/ml) per μg DNA. For transient transfection of plasmids, 2 μg total DNA/200 000 cells/35 mm dish were used. Expression was allowed to proceed for 24 h.

Cytosolic cell extracts

Cytosolic cell extracts were prepared as previously described (51).

Western blotting

Western blotting was performed according to (11) 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).

Immunoprecipitations and immunofluorescence

Immunoprecipitations and immunofluorescence were conducted as described (11).

TOP/FOP reporter assays

The TOPglow reporter consists of a tandem repeat of four TCF/LEF1 binding sites inserted in front of a TATA box, driving the expression of luciferase in a β-catenin-dependent manner. In the FOPglow reporter, the four binding sites are mutated to abolish the binding of TCF/LEF1. The internal control pUHC16.1 encoding the β-galactosidase was transiently transfected together with either FOP or TOP plasmids at a ratio 1:1. The ratio of pUHC16.1 to any additional plasmid was 1:2. The transcriptional activity measured 24 h post-transfection is defined as the ratio of TOP and FOP luciferase values normalized to the β-galactosidase values.

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Conflict of Interest statement. None declared.

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