Novel self-association of the APC molecule affects APC clusters and cell migration

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Summary

Truncation mutations in the adenomatous polyposis coli (APC) gene are responsible for familial and sporadic colorectal cancer. APC is a multifunctional protein involved in cell migration, proliferation and differentiation. The APC protein forms specific clusters in the cell periphery that correlate with sites of active cell migration. Little is known about the molecular mechanisms that govern these clusters. Here, we identify a novel interaction of an N-terminal region of APC with the extreme C-terminal 300 amino acids of APC and also with itself. The latter interaction is phospho-sensitive and is enhanced by 14-3-3 (YWHA) protein. These interactions modulate the clustering of APC at the ends of membrane protrusions. Overexpressing this domain or inhibiting 14-3-3 proteins disperses APC clusters and leads to decreased cell migration. Moreover, deleting this domain from full-length APC results in less-dynamic clusters compared with wild-type APC. Our data indicate that this newly identified region in the N-terminal third of APC contributes to the regulation of APC clusters, thus providing a molecular clue for how locally regulated phosphorylation events could mediate the dynamics of APC clusters and contribute to cell migration.

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Key words: Adenomatous, Cell migration, Polyposis coli

Introduction

Loss of the adenomatous polyposis coli tumour suppressor (APC) is intimately linked to colon cancer (Kinzler and Vogelstein, 1996; Polakis, 2000). Restoring the wild-type allele into an APC mutant cell line can lead to reversion of the transformed phenotype (Faux et al., 2004; Morin et al., 1996). APC is an essential component of the Wnt signalling pathway and is required for the formation of a cytoplasmic complex that phosphorylates ß-catenin and thus mediates its targeting to the proteasome for degradation (Bienz and Clevers, 2000; Fodde, 2002). Active Wnt signals disrupt this complex, leading to the stabilisation and accumulation of non-phosphorylated ß-catenin that binds to members of the TCF-LEF family of transcription factors and activities Wnt-dependent gene transcription (Fodde, 2002; van de Wetering et al., 2002).

Most human colorectal tumours carry mutations in the APC gene that result in the expression of truncated N-terminal APC fragments lacking sites required for the formation of the ß-catenin targeting complex so that intracellular ß-catenin is not regulated properly and the normal genetic programme is altered (Schneikert and Behrens, 2007). In addition, APC participates in several other cellular processes, including cytoskeletal regulation, so that loss or truncation of APC also directly affects cell migration and chromosome segregation (Dikovskaya et al., 2004; Green and Kaplan, 2003; Kita et al., 2006; Kroboth et al., 2007; Sansom et al., 2004). Consistent with an important role for APC in cytoskeletal architecture, APC forms a complex with other cytoskeletal proteins, including EB1 (MAPRE1), KAP3A (KIFAP3), ASEF (ARHGFE4), IQGAP and DLG (Iizuka-Kogo et al., 2005; Jimbo et al., 2002; Kawasaki et al., 2000; Su et al., 1995; Watanabe et al., 2004). However, what confers the highly selective localisation of APC clusters to only a few actively migrating membrane protrusions is not clear (Näthke et al., 1996b). Phosphorylation of APC by GSK3ß can cause APC to dissociate from microtubules and it is possible that APC inactivation at specific locations is involved in restricting the accumulation of APC to certain sites (Etienne-Manneville and Hall, 2003; Näthke et al., 1996b; Zumbrunn et al., 2001). The ability of APC to cluster at specific membrane protrusions requires its N-terminal domain, which binds to KAP3A and is also involved in the ability of APC to self-associate via N-terminal coiled-coil domains; however, this particular self-interaction is constitutive and more than one mechanism for self-association must be invoked to explain the formation of such large and dynamic clusters in specific areas of cells (Joslyn et al., 1993).

We previously found that N-terminal APC fragments similar to those expressed in tumour cells can directly bind to regions in the C-terminal domain of APC and that this particular interaction can modulate the protein interactions of isolated N-terminal fragments expressed in tumour cells (Li and Näthke, 2005). To further characterise APC self-interactions, and their regulation and function, we first identified smaller regions in the APC molecule that directly bind each other. Our experiments showed that the region in the N-terminal of APC directly C-terminal to the armadillo repeats (we named this region N3, see Fig. 1A) binds to the C-terminal 300 amino acids of APC (referred to as C3). Moreover, we found that the N3 region binds to itself. This particular self-association can be regulated by phosphorylation and is enhanced by the phosphorylation-dependent linker 14-3-3 (YWHA) in vitro. We also show that the overexpression of N3 causes the dispersal of APC clusters in cells and significantly decreases cell migration. Correspondingly, clusters formed by APC lacking the N3 region are less dynamic than those formed by wild-
type APC. Overexpressing isolated C3 fragments also led to the dispersal of APC clusters and APC lacking the C3 region formed less-dynamic clusters. However, these changes were less pronounced than those achieved with N3. Together, our data suggest that the newly identified self-association of the N3 region in APC contributes to the regulation of the formation and dynamics of APC clusters in membrane protrusions, and contributes to cell migration.

Results
Identification of domains within the N- and C-terminal regions of APC that bind to each other in vitro
The N-terminus of APC contains long stretches of coiled-coil domains as well as a set of seven armadillo repeats and it can dimerise via its initial 50 amino acids. However, this interaction is weak and additional sequences in the N-terminal region are implicated in this interaction (Day and Alber, 2000; Hanson and Miller, 2005; Joslyn et al., 1993; Su et al., 1993). We previously reported that N- and C-terminal regions of APC interact with each other, and that this association can modify the protein interactions of truncated N-terminal APC fragments found in colorectal cancer cells (Li and Näthke, 2005). Initially, we aimed to map more precisely the sites involved in the associations between N- and C-terminal regions of APC. To this end, three fragments were generated that spanned the N-terminal APC region (N-APC, aa 1-1018). These fragments were called N1, N2 and N3 and comprised residues 1-448, 449-781 and 782-1018, respectively (Fig. 1A).

Radioactively labelled versions of each of these fragments were generated by in vitro translation in the presence of 35S-methionine (Fig. 1B). The C-terminal APC region (C-APC, aa 2038-2843) was purified using a His-tagged bacterially expressed protein (Fig. 1C).

To investigate the binding between C-APC and N1, N2 or N3, purified C-APC was immunoprecipitated with an anti-C-APC antibody and incubated with radioactively labelled N-APC fragments. Binding of N-APC fragments was determined by autoradiography (Fig. 1D). To confirm that identical amounts of C-APC were present in each case, samples were immunoblotted with a monoclonal anti-C-APC antibody (Fig. 1D, lower panel). These experiments revealed that N2 and N3 specifically bound to C-APC in vitro. We did not detect specific binding of N1 to C-APC. This particular fragment strongly bound to the control affinity matrix and aggregated readily. The high background binding of the N1 fragment to the antibody beads lacking C-APC (Fig. 1D) is probably due to the high potential of this region to form extensive coiled-coils. This is reflected by its ability to dimerise via the initial 50 amino acids, which might further exacerbate its propensity to be extremely sticky; this prevented our ability to investigate the specific protein interactions of this fragment in vitro (Joslyn et al., 1993).

N3 and C3 are important for the interaction between the N- and C-terminal regions of APC
Next, we aimed to identify region(s) within C-APC that were involved in the interaction with N-APC. C-APC was divided into three fragments: C1 (aa 2035-2182), C2 (aa 2166-2547), containing the entire direct microtubule-binding site, and C3 (aa 2545-2843), containing the EB1- and PDZ-binding domains (Fig. 1A). Another version of C3, lacking the last 15 amino acids to abolish binding to PDZ domains and with Ile 2806 and Pro 2807 changed to serines...
to reduce binding to EB1, was also generated and named C3m (Honnappa et al., 2005; Marfatia et al., 2000). All C-APC fragments were expressed as His-tagged fusion proteins in Escherichia coli and were purified using Ni-agarose (Fig. 1B). In addition, vectors used for the expression of GFP-tagged versions of these C-APC fragments in mammalian cells were prepared. To measure the ability of these fragments to bind to N-APC, the N-terminal APC fragment expressed in SW480 cells was immunoprecipitated from extracts of cells transiently transfected with the GFP-tagged versions of the C-APC fragments (Fig. 2A). Co-precipitated C-APC fragments were then detected using an anti-GFP antibody (Fig. 2A, bottom panel). Only C-APC, C3 and C3m, but not C1 or C2, bound to the endogenous N-APC (Fig. 2A).

Having established that N2 and N3 could bind to C-APC in reticulo-lysate in vitro (Fig. 1D), we needed to determine whether both of the regions were required for this interaction. The APC fragment in SW480 terminates at residue 1338, and thus contains both N2 and N3, whereas the fragment in Colo320 terminates at residue 811 and thus lacks the second half of N3. To compare binding of the different endogenous N-terminal APC fragments to C-APC, the same panel of C-APC fragments was transfected into SW480 and Colo320 cells. Endogenous N-terminal APC was immunoprecipitated from both cell types (Fig. 2A,B). Both cell types expressed the C-APC fragments, albeit at slightly different levels (Fig. 2A,B). However, C-APC, C3 and C3m did not associate with the shorter N-terminal APC that is present in Colo320 cells (Fig. 2B). This suggested that N3 is required for the association between N- and C-APC in vivo.

To further confirm an interaction between C3 and N3, we measured the association between purified C3 and in vitro translated 35S-labelled N1, N2 and N3 (as in Fig. 1B,C). Purified C3 was bound to polyclonal anti-C-APC antibody that was immobilised on beads. This was then incubated with in vitro translation reactions containing N-APC fragments. Bound N-APC fragments were detected by autoradiography (Fig. 2C). Consistent with the in vitro binding data shown in Fig. 1, C3 specifically bound both N2 and N3, but not N1. This suggested that N3 and C3 are the regions directly involved in the interactions between N- and C-terminal APC domains. Consistent with this idea, N-APC in Colo320 cells, which lacks a substantial portion of N3 but still contains N2, did not bind to C3. At this time, we cannot formally eliminate the possibility that the N3-C3 interaction is indirect and involves additional proteins present in cells and reticulo-lysates.

**Self-association of N3 is enhanced by phosphorylation**

Recent studies confirmed that the first 58 amino acids of APC, which are involved in coiled-coil-mediated dimerisation of the protein, are important for the dominant effect of APC on mitosis. However, these studies also implicated residues 768-1390 in this effect, suggesting that there are two domains that contribute to the activity of N-APC (Green et al., 2005; Tighe et al., 2004). To investigate whether the N3 region, which is contained within residues 768-1390, also contributes to the ability of APC to self-associate, we expressed N3 as a GFP fusion protein in SW480 and Colo320 cells. We then immunoprecipitated the endogenous, truncated N-terminal APC fragments from these cells with an antibody that recognises residues 44-140 (and thus does not bind to N3) and probed samples of the bound proteins with an antibody against GFP to determine whether N3 could interact with the N-terminal fragments in these two different tumour cells. As expected, N3 only bound to the N-terminal APC fragment in SW480 cells, but not in Colo320 cells, which only express APC that lacks most of the regions spanned by N3 (Fig. 3A). The specificity of the interaction of N3 with N-APC was further confirmed by our finding that N2 did not co-precipitate with N-APC in SW480 cells (supplementary material Fig. S1). The ability of N3 to bind itself directly was suggested by our finding that purified N3 migrates as a dimer on native PAGE (supplementary material Fig. S1). However, we cannot exclude the possibility that binding of N3 to N-APC in reticulo-lysates and in cells is indirect and involves other proteins.

Phosphorylation renders the APC molecule more stable towards proteases and is also involved in enhancing its binding to β-catenin (Li and Näthke, 2005; Rubinfeld et al., 1996). To determine whether the interactions we identified are affected by
phosphorylation, we compared the binding of C-APC, C3 or N3 to N-terminal APC, isolated from SW480 cells using an anti-N-APC antibody, before and after treatment with λ-phosphatase (Fig. 3B). The blot in Fig. 3B shows a visible shift in the electrophoretic mobility of N-terminal APC after λ-phosphatase treatment, confirming that APC is normally phosphorylated in cells and was successfully dephosphorylated by the treatment (Fig. 3B). Next, purified His-tagged C-APC, C3 or N3 (Fig. 1C) were added to the isolated N-APC and their interaction measured by immunoblotting bound material with an anti-His antibody. Using this in vitro approach, we found that the dephosphorylation of N-APC decreased its binding to N3 (Fig. 3B). However, the binding of N-APC to C-APC or C3 was not consistently affected (Fig. 3B). (The slight increase in the amount of C3 that was bound after treatment with λ-phosphatase was not reproducible.)

There are two possibilities for how phosphorylation could change N3 binding to N-APC: (1) it could induce a conformational change or (2) it could induce the recruitment of cofactor(s) to enhance the interaction. We explored this second possibility further based on the presence of a predicted 14-3-3-binding site in the N3 region of APC (Swiss prot, accession number: p25054, with sequence ESNRRCMPYAKLE, where the underlined S948 marks the predicted phospo-sensitive, 14-3-3-binding site). 14-3-3 proteins are abundant proteins that function as phospho-serine- or phosphothreonine-binding modules (Wilker and Yaffe, 2004). They bind to specific phosphorylated residues on target proteins to modify their function and interactions (Mackintosh, 2004). To establish whether this phospho-sensitive linker contributes to the self-association of APC via N3, we compared the binding of purified C-APC, C3 and N3 to N-APC isolated from SW480 cells in the absence and presence of isolated GST-tagged 14-3-3 proteins (Fig. 3C). Indeed, binding of N3 to N-APC was strongly enhanced by the presence of 14-3-3 protein (Fig. 3C). By contrast, binding of C-APC or C3 to N-APC was not consistently altered (Fig. 3B,C). Furthermore, we confirmed that N-APC in SW480 cells, but not in Colo320 cells, strongly bound to 14-3-3 proteins in a phospho-dependent manner (Fig. 3D). When N-APC immunoprecipitated from these two cell lines was treated with λ-phosphatase and subsequently incubated

Fig. 3. Binding of N3 to itself is regulated by phosphorylation. (A) Equal amounts of proteins from cell lysates of SW480 or Colo320 cells transfected with GFP-N3 or GFP alone were prepared and immunoprecipitated with a monoclonal anti-N-APC antibody. Total lysates (left panels) and proteins bound to the immunoprecipitated material (right panels) were probed with an anti-GFP antibody. Only the longer N-APC in SW480 cells bound to GFP-N3. (B) Endogenous N-APC was immunoprecipitated from SW480 cells and treated with λ-phosphatase (+) or control buffer (–). Purified His-tagged C-APC, C3 or N3 were added and bound proteins were detected using a monoclonal anti-His antibody. Only the binding of N3 was significantly inhibited when N-APC was dephosphorylated. In addition, the precipitated material was probed with a monoclonal anti-N-APC antibody to confirm that equal amounts of N-APC were recovered in each case (upper panel) and also to illustrate that treatment with λ-phosphatase increased the electrophoretic mobility of the N-APC in these cells, confirming that the N-terminal third of APC is normally phosphorylated in cells. (C) Immunoprecipitated N-APC from SW480 cells was incubated with purified His-tagged C-APC, C3 or N3 in the presence (+) or absence (–) of GST-tagged 14-3-3 protein. Bound C-APC, C3 and N3 were detected using a monoclonal anti-His antibody, and the presence of 14-3-3 was confirmed with a polyclonal anti-GST antibody (lower panel). (D) Immunoprecipitated endogenous APC from SW480 or Colo320 cells was incubated with purified GST–14-3-3 in the presence (+) or absence (–) of λ-phosphatase. Only the N-APC from SW480 cells bound 14-3-3 proteins strongly and this binding required phosphorylation of N-APC, consistent with the idea that the N3 region (absent in the APC in Colo320 cells) is required for this interaction. (E) Immunoprecipitated endogenous APC from SW480 cells was treated with λ-phosphatase or control buffer before purified GST–14-3-3ζ was added to the beads. After washing, purified N3 was added to each sample. Bound proteins were detected with the indicated antibodies. The control sample did not include the cell lysate. Both phosphorylation and the binding of 14-3-3 enhance the ability of N3 to bind N-APC.
with purified GST-tagged 14-3-3, only the longer N-APC from SW480 cells bound 14-3-3 and this interaction was inhibited by λ-phosphatase. Further comparison of the binding of N3 to N-APC in response to changes in dephosphorylation and to the presence or absence of 14-3-3 protein in the same samples confirmed that both phosphorylation and the presence of 14-3-3 protein enhance this interaction (Fig. 3E) so that phosphorylated N-APC binds 14-3-3 protein much better, and that this combination recruits measurably more N3. The ability of N3 to directly bind 14-3-3 proteins in a phospho-sensitive manner was further confirmed in overlay assays (supplementary material Fig. S2).

The N3 region disperses APC clusters and decreases cell migration

APC accumulates in large peripheral clusters at the end of microtubules (Näthke et al., 1996a). Given the specific localisation of these clusters and their dynamic nature, it is highly likely that regulated self-association of APC is involved in its ability to form these large clusters. To determine how changes in the newly identified intracellular interaction between the N3 and C3 regions of APC affected these clusters, we measured the effect of overexpressing N3 and C3 on clusters of endogenous APC in MDCK cells (both N3 and C3 are predicted to compete with endogenous APC for binding to itself) (Näthke et al., 1996a). We first established a set of standard APC distributions that we classified, as shown in Fig. 4A, as (a) cluster, (b) dispersed cluster and (c) dispersed. We then counted the number of cells that showed each of these distributions for endogenous APC in cells that were transfected with GFP-tagged N3 or C3, or GFP alone as control (Fig. 4Ba). Endogenous APC was detected with an antibody that does not bind to N3 or C3 (anti-M-APC). We found that the expression of N3 caused a significant reduction in the number of cells in which endogenous APC clustered tightly in membrane protrusions. Instead, more cells had dispersed APC clusters (Fig. 4Ba). The expression of C3 produced a much less significant effect, suggesting that N3-N3 interactions were more involved in the clustering of APC. Consistent with this idea, when full-length APC (GFP-tagged) was co-transfected with GST-tagged N3 in transient

Fig. 4. APC-cluster formation in MDCK cells is inhibited by expression of N3 or by the inhibition of 14-3-3 proteins.
(A) Representative images of the different types of endogenous APC (a–c) or GFP-APC protein (a’–c’) clusters used to categorise cells: (a) cluster, (b) dispersed cluster, (c) dispersed. APC is green; tubulin is shown in red. (B) APC-cluster formation in cells was scored using a total of ~100 cells randomly selected from three independent experiments. (a) The localisation of endogenous APC in MDCK cells was scored in cells transiently transfected with N3, C3 or GFP. Expression of N3 caused a marked decrease in the number of cells with clustered APC and led to more cells with dispersed APC, whereas C3 had a more moderate effect. (b) The localisation of GFP-tagged full-length APC in MDCK cells was compared in cells co-transfected with GST or with GST-N3. Again, the presence of N3 led to a decrease in clustering of GFP-APC. Similar data was obtained in PTK2 cells (supplementary material Fig. S4). (c) The localisation of endogenous APC was scored in cells treated with a phospho-peptide (inhibitor) or the non-phospho version of the same peptide (control). Inhibition of 14-3-3 caused a reduction in the number of cells with clustered APC. (d) Clusters formed by GFP-tagged APC lacking N3 (GFP-APCΔN3) and GFP-APC were scored as above. GFP-APCΔN3 forms tighter clusters compared with GFP-APC. Similar data was obtained in PTK2 cells (supplementary material Fig. S4). (C) Images of live cells expressing GFP-APC together with mCherry-N3 (a) or mCherry-C3 (b) reveal enrichment of N3 in APC clusters. C3 is not enriched in the clusters, but is also not excluded from them.
expression experiments, there was a drop in the number of cells with GFP-APC in tight clusters (Fig. 4Bb and supplementary material Fig. S4A). MDCK cells stably expressing the N3 fragment also had more dispersed APC clusters than GFP-expressing control cells (supplementary material Fig. S3). Importantly, treating cells with a phospho-peptide that inhibits 14-3-3 proteins led to a similar effect (Fig. 4Bc), suggesting that 14-3-3 proteins contribute directly or indirectly to the clustering of APC (Moorhead et al., 1999; Pozuelo Rubio et al., 2003).

To determine whether the N3 region contributes to APC clusters in the context of the full-length APC molecule, we also compared clusters formed by full-length APC to those formed by full-length APC lacking the N3 regions (APCΔN3). Consistent with our hypothesis that the N3 region contributes a regulatory site for cluster assembly, clusters formed by APCΔN3 tended to be less dispersed and more coherent. This was observed in MDCK and in PTK2 cells (Fig. 4Bd and supplementary material Fig. S4B). Consistent with an effect of N3 on APC clusters, we could detect N3 tagged with mCherry fluorescent protein in APC clusters (Fig. 4Ca). Please note, we chose a tight cluster to illustrate this colocalisation because the high background of cytoplasmic N3 made it difficult to see N3 enriched on smaller, dispersed APC clusters, which were encountered more frequently (see supplementary material Fig. S4A) in these cells. C3 was not enriched at APC clusters, although it was not excluded from these regions (Fig. 4Cb).

APC participates in several cellular processes, including cytoskeletal regulation, so that loss or truncation of APC directly affects cell migration and chromosome segregation (Dikovskaya et al., 2004; Dikovskaya et al., 2007; Green and Kaplan, 2003; Kitu et al., 2006; Kroboth et al., 2007; Sansom et al., 2004). To determine whether dispersing APC clusters correlated with changes in cell behaviour, using a scratch-wound assay we measured the migration of MDCK cells stably expressing N3 (Fig. 5). When N3 was stably expressed in MDCK cells, migration decreased by 40% compared with MDCK cells stably expressing GFP, as shown in the box plots in Fig. 5B. These data suggest that the ability of APC to interact with itself via the N3 region is important for the formation of dynamic APC clusters, which in turn contribute to directed migration of epithelial cells.

To further establish how APC-APC interactions between the N3 and C3 regions contribute to the dynamics of APC clusters, we compared the behaviour of clusters formed by GFP-APC, GFP-APCΔN3 and GFP-APCΔC3 by measuring the total fluorescent intensity contained in individual clusters over time and comparing these values to the initial intensity (Fig. 6). We chose this method rather than conventional fluorescent recovery after photobleaching (FRAP) because reorganisation of the clusters was faster than recovery of the fluorescent signal, making it impossible to monitor the same structure over time by FRAP (see supplementary material Movies 1-3). Our analysis revealed that clusters formed by GFP-APC were more dynamic than those containing GFP-APCΔN3, as indicated by the larger changes in the clusters formed by GFP-APC (Fig. 6D). Clusters formed by GFP-APC lacking the C3 region were also less dynamic, but this effect was less pronounced than for those formed by GFP-APCΔN3. Together, these data confirm that interactions of the N3 region in APC are important for the dynamics of APC clusters. Our data suggest that the N3 region provides a molecular means for regulating the association and dissociation of APC in clusters and thus introduces dynamics into cluster assembly.

**Fig. 5.** MDCK cells stably expressing N3 migrate more slowly. (A) MDCK cells stably expressing GFP or GFP-N3 were imaged at the time of induction of a scratch into a monolayer (shaded blue) and 8 hours later (shaded grey). Cells expressing N3 migrated more slowly. (B) The migration of MDCK cells stably expressing N3 or GFP was measured over 8 hours and was significantly reduced compared with GFP-expressing control cells. Data from four independent experiments using two different clones are shown. Asterisk indicates statistical significance (*P*<0.001). (C) Lysates from MDCK cells stably expressing GFP-N3 or GFP corresponding to equal amounts of protein were blotted with anti-GFP antibodies to show the relative expression levels of these proteins.

**Discussion**

Our data add new information about how peripheral clusters of APC are regulated and identify the N3 region, which lies immediately adjacent to the armadillo repeats, as the domain that contributes importantly to this process. Furthermore, we found that the final 340 amino acids of APC, C3, bind to the N3 domain and that this interaction might also play a role in APC clustering. These data establish the N3 region of APC, to which no function had been assigned previously, as an important integrator of APC self-interactions.

We previously reported that the microtubule-binding region (represented by C2) was required for the interaction between N- and C-APC because a C-terminal fragment called CA2, which lacks this site, does not bind to N-APC (Li and Näthke, 2005). Our new data suggest that the C3 region, not C2, harbours the site involved in the association between N-APC and C-APC because isolated C3 but not C2 bound to N-APC (Fig. 2A). It is possible that, in the CA2 fragment, aberrant folding affected the ability of C3 to bind N-APC. Alternatively, both C2 and C3 might contribute to the interaction between C-APC and N-APC, but C2 might not be sufficient on its own. This idea is confirmed by the finding that C-APC bound to N-APC better than C3 did, as indicated by the observation that, relative to their expression levels, proportionally more C-APC than C3 was recovered bound to N-APC (Fig. 2A).

Nonetheless, our data suggest a direct interaction between N-APC and C-APC that can be mediated by the C3 region independently of the EB1 or PDZ proteins, because C3m, which lacks both of these interaction sites (Honmappa et al., 2005), could still be co-
precipitated with N-APC. Although our data also showed that isolated N2 could bind to C3 in vitro, this interaction was not detected in cells. This raises the possibility that other binding partners of APC that associate with N2 (such as IQGAP, B56, KAP3A and ASEF) might create a steric hindrance. It is, of course, formally possible that proteins in the reticulo-lysate mixture contributed to this effect.

Importantly, our new results suggest that APC self-association is mediated not only by the extreme N-terminal residues of APC, as shown previously, but also by residues contained in the N3 fragment. Our experiments suggest that self-association of N3 is regulated by phosphorylation and that the phospho-sensitive linker 14-3-3 can enhance this interaction, at least in vitro (Fig. 3C). Direct binding of 14-3-3 proteins to N3 was suggested by our finding that these fragments bound purified 14-3-3 protein in overlay assays (supplementary material Fig. S2). Based on these data, we propose that the N3 region is involved in the dynamics of APC clusters and contributes to the association and disassociation of the APC.

**Fig. 6.** Clusters formed by APCΔN3 are less dynamic. (A–C) Images of clusters containing GFP-APC (A), GFP-APCΔN3 (B) or GFP-APCΔC3 (C) were recorded every 100 seconds to monitor shape and intensity changes over time. The summed intensity in each cluster was recorded and two examples of different types of clusters at 0, 300, 600 and 900 seconds are shown. Please note that these images show projections of the total summed intensity, not maximum intensity projections. This makes their appearance more fuzzy, but allows the changes in total intensity to be quantitated and visualised accurately. The appearance of clusters formed by GFP-APC changed noticeably more than of those formed by GFP-APCΔN3, whereas changes by those formed by GFP-APCΔC3 were intermediate. (D) Total fluorescent intensity in selected clusters was measured and recorded over time (seconds). The intensity in each cluster relative to that at the start is plotted for each time point to show that clusters formed by GFP-APCΔN3 are less dynamic than those formed by GFP-APC or GFP-APCΔC3.
molecule in these structures. Expressing N3 or inhibiting 14-3-3 proteins led to the dispersal of the clusters but did not abolish smaller APC puncta (Fig. 4). Correspondingly, clusters formed by APC lacking N3 (APCN3) tended to be less dispersed and less dynamic (Fig. 4D and Fig. 6). These data suggest that this particular region of APC is involved in the assembly and disassembly of higher order APC structures that affect the local concentration and dynamics of these structures. This in turn might be important for the concentrated and highly localised effect on microtubules in migrating protrusions.

Our observation that C3 might also contribute to APC clusters, albeit to a lesser extent, is more difficult to interpret because known binding partners of C3, most importantly EB1, could also be affected by changes in this site. However, we found that the expression of C3 did not cause a detectable change in the localisation of EB1 (data not shown), suggesting that the less-severe effects we observed might be due to its contribution to APC-APC self-interactions. This conclusion is also supported by the lack of enrichment of isolated C3 to APC clusters (Fig. 4C).

In summary, our studies identified a novel self-interaction site in the APC tumour suppressor protein that can contribute to the regulation of this multifunctional protein. The region directly adjacent to the armadillo repeats (N3) binds to itself, and this interaction affects the formation and behaviour of peripheral APC clusters. This self-association can be regulated by phosphorylation and is enhanced by 14-3-3 proteins. Disrupting this particular interaction leads to the dispersal of APC and causes changes in cell migration. These findings provide novel insights into the regulation of the APC protein, and provide a molecular explanation for how specifically localised phosphorylation events that have been proposed to stimulate the effects of APC on microtubules could contribute to the localised, dynamic clustering and activation of APC in actively migrating areas of cells (Hall, 2005). Indeed, a reduction in cell migration has been reported in epithelial cells in Min mice, which are heterozygous for APC (Mahmoud et al., 1997; Oshima et al., 1997). Moreover, our data have implications for how interactions between N-terminal APC fragments expressed in early tumour cells and full-length APC protein can contribute to deregulation in early tumour cells, when cells are still heterozygous for the APC protein. Our data suggest that, in this situation, the interaction(s) between N-terminal APC fragments and full-length APC protein might also interfere with the remaining wild-type copy of the protein and this might compromise the upward migration of intestinal epithelial cells out of the crypt.

Materials and Methods

Antibodies and protein reagents

Antibodies used in this study were: mouse monoclonal anti-N-APC (ALI, epitope: aa 45-140) that does not bind N3 (Cancer Research UK), mouse monoclonal anti-C-APC (epitope: aa 2130-2420; Cancer Research UK), polyclonal anti-C-APC (epitope: aa 2550-2810) (Midgley et al., 1997), rabbit anti-M-APC polyclonal antibody (Näthke et al., 1996a), polyclonal anti-GFP (AbCam), monoclonal anti-GFP (Roche), anti-GST (Amersham), anti-His (Quagen), anti-tubulin and DM16 (Sigma). Lambda- phosphatase (δ-iso) was from New England Biolabs.

Cell culture and transfection

SW480 cells were cultured as described previously (Li and Näthke, 2005). Colo320 cells were cultured in RPMI 1640 (L-glutamine, Invitrogen, UK) under standard conditions. MDCK cells were maintained in Dulbecco’s modified eagles medium (DMEM, Sigma) supplemented with 1 g/l sodium bicarbonate. PTK2 cells were maintained in DMEM/F12 HAM supplemented with 2 mm L-glutamine. All media contained 10% fetal calf serum (Sigma), penicillin and streptomycin (100 U/ml, GIBCO). Cells were transfected using Polyfect transfection reagent (Quagen, GmBH for SW480 and Colo320, Lipofectamine 2000 (Invitrogen, UK) for MDCK cells, and Eugene 6 (Roche, Mannheim, Germany) for PTK2 cells, according to the manufacturer’s instructions.

Stable cell lines and migration assays

All the N-APC fragments were cloned into the pEGFP3 vector, which contains a neomycin-resistance cassette to permit G418 selection (1 mg/ml; Clontech Laboratories). For scratch-induced cell migration, 10⁶ cells were seeded into six-well dishes and grown for 36 hours. Monolayers were scratched with a pipette tip to create a large gap and then recorded using time-lapse microscopy (LEICA DMIRB) for 8 hours. The distance moved by the migrating front was measured as follows: images of the moving front at time 0 and 8 hours later were opened in Velocity (Improvision, Volocity 4.0) and, for each slide, five points were marked on the free edge, equally spaced along the width of the slide (600 μm). The distance between the moving front at 0 and 8 hours was then measured for each point. The data of 90 measurements are represented in a box plot (Fig. 5B). Results shown are from four independent experiments using two independent clones. Comparison between each group vs GFP controls was performed using SPSS statistics software.

Plasmids

DNA sequences encoding full-length APC, APCN3 and APCΔC were cloned into pEGFP3, and N1, N2, N3, C1, C2, C3 and C3m (see Fig. 1) were cloned into pEGFP3 to introduce an N-terminal GFP tag (Fig. 1A). N3 fused to GST was cloned into the pET22 vector. N3 and C3 fused to mCherry were generated by cloning N3 and C3 into pmCherry-C1 (Shaner et al., 2004). All plasmids for in vitro translation were prepared using the PhEl2.4b vector. A series of His-tagged APC fragments for bacterial expression were constructed using pET28a. All recombinant plasmids were confirmed by restriction analysis and sequencing.

Immunofluorescence microscopy and quantitation of APC clusters

MDCK cells were seeded at 5×10⁵ cells per well into six-well plates containing collagen-coated coverslips (Sigma). Cells were allowed to adhere and grow for 24 hours before transfection with 1-3 μg of the appropriate APC constructs. 48 hours after transfection, cells were fixed with −20°C methanol for 5 minutes. Coverslips were treated with 4% donkey serum in PBS, 0.1% Triton X-100 for 1 hour. Cells were stained with antibodies against APC (anti-M-APC, diluted 1:500) (Näthke et al., 1996a) and tubulin (1:300). After washing with PBS, 0.1% Triton X-100, appropriate fluorescently conjugated secondary antibodies were applied (dilution 1:120; Molecular Probes) and DNA was stained with 4’-diamidino-2-phenylinole (Sigma). Fluorescence images were obtained using a Delta Vision restoration microscope (Applied Precision, Issaquah, WA). A total of approximately 100 cells were randomly selected from three independent experiments and the status of APC clusters in each cell was determined for each sample. Results are expressed as mean ± standard deviation. Examples for each category (cluster, dispersed cluster and dispersed) were defined as shown in the images in Fig. 4A.

To follow the dynamics of clusters formed by GFP-APC or GFP-APCΔN3 and GFP-APCΔC, PTK2 cells transfected with the corresponding DNA construct were imaged at 100-second intervals on a DeltaVision Spectris microscope (Applied Precision, Issaquah, WA). Images were recorded with an Olympus 100X/1.40, Plan Apo, IX70 lens and captured with a Photometric COOLSnap HQ camera (Roper Scientific, Tuscon, AZ) (gain=0.50X, speed: 10,000 kHz, temperature setting: −30°C) as enabled by the DeltaVision software Softworx. Images were analyzed using Velocity as follows. The outline of a cluster (only clusters ≤4 μm were used) was delineated at each time point and the total summed intensity of the entire cluster was measured for the entire image stack. Background was measured in an area free of cells on the same slide and subtracted. The total intensity at each time point was expressed as a percentage of the intensity at the beginning.

Immunoprecipitation and western blotting

Cell lysates were prepared in MECB buffer (50 mM Tris, 100 mM NaCl, 5 mM EDTA, 5 mM EGTA, 40 mM β-glycerophosphate; 0.5% NP40 containing a cocktail of protease inhibitors (10 μg/ml leupeptin, pepstatin A and chymostatin A), 50 mM sodium fluoride, 100 μM sodium orthovanadate. To measure the binding of different C-APC fragments to N-APC in cells, 1 mg of cell lysate from cells transfected with the C-APC fragments was immunoprecipitated with 10 μg of monoclonal anti-N-APC (α-22a) that had been pre-bound to 100 μl of 50% protein G-Sepharose bead slurry (Cancer Research UK) by gently agitating the mixture at 4°C for 2 hours. The complexes were washed three times with MECB buffer. To measure the binding of purified N- and C-fragments to each other, purified C, C3 or N3 were added to N-APC immunoprecipitated from cell lysates as above for 1-5 hours incubation. The proteins were separated by SDS-PAGE gels and blotted to nitrocellulose. Western blotting was performed as described previously (Li and Näthke, 2005). Detection was either with horseradish-peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (Pierce, Tottenhall, UK) or...
fluorescently labelled secondary antibodies using a LiCor Odyssey (Bioscience) for detection.

Association of C and C3 fragments with in vitro translated N-APC fragments

His-tagged C-APC or C3 was expressed in E. coli and purified on a Ni-agarose column.

Movies

Movies were acquired as follows. PTK2 cells were kept in an environmental chamber at 37°C in 5% CO2-independent media ( Gibco , supplemented with Pen/Strep FCS and L-Glu, as described in the Materials and Methods). Cells were transfected and seeded into 35-mm dishes for 4 hours. Images corresponding to a 14-3-3-binding consensus sequence with or without phosphorylation at the relevant serine, RAASFP/AA (inhibitor) or RAASAP (control), linked to 5-carboxyfluorescein were added to a final concentration of 100 μM/ml for 3 hours. Cells were fixed and stained for APC and tubulin.

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