Characterization of a 60S complex of the adenomatous polyposis coli tumor suppressor protein

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Abstract

The tumor suppressor protein adenomatous polyposis coli (APC) is a multifunctional protein with a well characterized role in the Wnt signal transduction pathway and roles in cytoskeletal regulation and cell polarity. The soluble pool of APC protein in colon epithelial tumor cells exists in two distinct complexes fractionating at ∼20S and ∼60S in size. The 20S complex contains components of the β-catenin destruction complex and probably functions in the Wnt pathway. In this study, we characterized the molecular nature of the 60S APC-containing complex by examining known potential binding partners of APC. 60S APC did not contain EB1 or diaphanous, proteins that have been reported to interact with APC and are implicated in microtubule plus end stabilization. Nor did the two other microtubule associated proteins, MAP4 or KAP3, which is thought to link APC to kinesin motor proteins, associate with the 60S complex. Minor fractions of α-tubulin, γ-tubulin and IQGAP1, a Rac1 and CDC42 effector that interacts with APC, specifically associated with APC in the 60S fraction. We propose that 60S APC is a discrete high molecular weight complex with a novel function in cytoskeletal regulation in epithelial cells apart from its well established role in targeting catenin destruction or its proposed role in microtubule plus end stabilization.

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1. Introduction

The function of adenomatous polyposis coli (APC) as a “gatekeeper” gene in colon carcinogenesis is well established [1–4]. The APC gene product is a 312 kDa protein which has been proposed to play a role in a wide variety of cellular processes including the Wnt signaling pathway, cell cycle regulation, microtubule assembly, cell fate determination, cell adhesion and migration, chromosomal stability and apoptosis [5,6]. The best characterized biological function of APC protein lies in its ability to downregulate β-catenin, a key player in cell adhesion and in signal transduction in Wnt signaling pathway [7–10]. APC binds to the scaffold protein axin [11,12], which binds GSK3β and in turn mediates phosphorylation of β-catenin [13] thereby targeting β-catenin for degradation by proteosomes [14]. The tumor suppressor function of APC resides in its ability to bind axin and downregulate β-catenin and this function is lost in many colorectal cancer cells carrying APC mutation [14]. Studies on Drosophila, Xenopus and Caenorhabditis elegans have also revealed that APC has a role in the normal functioning of the Wnt/β-catenin signaling pathway [15–18].

APC has also been shown to interact with microtubules and regulate their dynamics in several different ways [19,20]. Endogenous full length APC localizes to some but not all growing microtubule plus ends in subconfluent epithelial cells [21,40], but when over expressed APC protein distributes along the microtubules and moves along a subset of microtubules towards the plus end [19,20,22]. Movement of APC along the microtubules can be attributed to its ability to interact with kinesin superfamily of plus end directed motor proteins KIF3a–KIF3b, through its association with KAP3 protein [23]. APC binds to microtubules either directly through its basic amino acid rich region near the C-terminus or indirectly through EB1, a microtubule end binding protein [24]. The direct microtubule binding region of APC appears to...
be important for APC’s involvement in microtubule stabilization [19,21,25] and microtubules that are associated with APC were found to be resistant to drug induced depolymerization both in vivo and in vitro. However the C-terminal APC fragment lacking the direct microtubule binding domain can promote microtubule assembly in presence of EB1 in vitro [24]. Another study has also shown that APC-EB1 complex at the microtubule plus ends interacts with formin protein diaphanos (mDia) and functions downstream of lysophosphaticid acid (LPA)-Rho-mDia signaling pathway [26]. This complex may contribute to microtubule stabilization either by directly capping microtubule plus ends or indirectly by contributing to other factors that may cap the plus ends in migrating cells [26]. It has also been suggested that association of APC with plus end of microtubule is essential for cell polarization and migration in astrocytes [27].

Another proposed role of APC resides in its ability to bind to IQGAP1, a protein involved in cytoskeletal organization. APC interacts with IQGAP1 and form a tripartite complex with activated Rac1 and Cdc42 and this complex has been proposed to link the actin cytoskeleton with microtubule dynamics during cell polarization and directional migration [28].

Although APC has been shown to play roles in many cellular processes and interact with a variety of proteins, very little attention has been paid to understand biochemical nature of APC complexes present in the cell. Analyses of the interaction of components of destruction complex in Wnt signaling pathway [8,12,29,30] suggest that it functions in cytosol. APC has also been shown to localize to the plasma membrane, perhaps colocalizing with apical plasma membrane [31], cortical actin or adherens junction [32]. Analysis of the subcellular distribution of APC and the destruction complex in polarized human colon epithelial tumor cells carried out in our laboratory revealed that the cytosolic pool of APC fractionated into two high molecular weight complexes of ~20S and ~60S in size [31]. Most of the cellular axin cofractionated with the 20S fraction and none of the components of destruction complex fractionated with the 60S fraction of APC raising the question of its function in cells. In order to understand the function of the 60S APC complex, we decided to determine whether it contains any known APC binding partners. We employed subcellular and protein fractionation as well as immunoprecipitation analyses to identify the proteins associating with APC in the 60S complex.

2. Materials and methods

2.1. Cell fractionation

The colorectal cancer cell line HCT116 was obtained from American Tissue Culture Center and maintained in McCoy’s 5a media containing 10% FBS. Cells were grown in the media until they were completely confluent. All cell fractions, as outlined in Fig. 1, were carried out at 4 °C. Confluent monolayer cells were washed briefly with phosphate buffered saline (PBS) and harvested by scraping in PBS and pelleting at 500 g for 5 min. Pelleted cells were resuspended in hypotonic lysis buffer containing 30 mM HEPES, pH 7.8 and 10 mM KCl and incubated for 30 min in ice. All the buffers contained the following protease inhibitors at the indicated concentrations: PMSF, 0.017 mg/ml; leupeptin, 0.002 mg/ml; aprotinin, 0.004 mg/ml; antipain, 0.01 mg/ml; benzamidine, 0.05 mg/ml; ST inhibitor, 0.01 mg/ml; iodoacetamide, 0.1 mg/ml. Cells were homogenized in a dounce homogenizer and the nuclei and the cell debris were removed by centrifugation at 50,000 rpm for 30 min. 1 M KCl was added to post nuclear fractions (PNF) to bring up the salt concentration to isotonic level (140 mM). PNFs were subjected to high speed centrifugation at 100,000 × g for 1 h to remove particulate and membrane fractions. Supernatant containing the soluble proteins (S100) was collected for further experiments. To pellet the 60S fraction (60S pellet) from the soluble pool of proteins, the S100 fraction was spun at 55,000 rpm for 48 min in a Beckman Coulter TLS55 rotor. The supernatant from the centrifugation was then used as the “20S” sample for following immunoprecipitation experiments.

2.2. Velocity sedimentation analysis

Aliquots of either the S100 or the 60S pellet containing 1.5–2 mg total protein was loaded on top of 4.5 ml of a continuous 10–40% sucrose gradient that was layered over 0.5 ml of a 66% sucrose cushion. The gradients were spun at 55,000 rpm in a Beckman Coulter SW55 rotor for 4.5 h. Fractions were collected and the proteins were precipitated with 10% trichloro acetic acid (TCA) for analysis by western blotting. Catalase (4–5S pool as monomer derived from 11.5S (trimer pool) and thyroglobulin (19S) were spun in parallel gradients to determine the sedimentation coefficients below 20S. To estimate S values >19S, a software program for the calculation of velocity gradients was used based on a method developed by McEwen [33].

2.3. Equilibrium density gradient centrifugation

To analyze whether the 60S pool of APC has any potential membrane association, the 60S APC pellet was resuspended in a 55% sucrose solution (in buffer A: 100 mM NaCl, 20 mM Tris–HCl, pH 7.8, 5 mM EDTA) and loaded onto 0.5 ml of a 66% sucrose cushion. 5 ml of a continuous 20–50% sucrose gradient was poured on top (in buffer A). The samples were spun in a Beckman Coulter SW 55Ti rotor at 45,000 rpm for 16 h at 4 °C. Fractions were analyzed by western blotting for APC.
2.4. Immunoblotting

For the detection of APC, samples were separated on 3% agarose gels under denaturing conditions and transferred by capillary transfer to nitrocellulose membrane overnight as described [34]. Western blots were performed as described [15]. Proteins were separated on 10% SDS polyacrylamide gel for the detection of tubulins, EB1 and actin and on 7.5% gel for the detection of E-cadherin, diaphanous, MAP4, KAP3 and IQGAP1, and then transferred to nitrocellulose membrane by semidry transfer. The following primary antibodies were used: APC, anti-APC COOH terminus (Ab-2, mouse IgG1; Oncogene Research Products), α-tubulin, monoclonal anti-α-tubulin (DM1A), γ-tubulin, monoclonal anti-γ-tubulin (GTU88), monoclonal anti actin (AC-40) and MAP4 (Sigma-Aldrich Inc), monoclonal anti-EB1, diaphanous, (p140mDia) and KAP3A (BD Transduction Laboratories). IQGAP1 MAb was kindly provided by Dr. George Bloom (University of Virginia, Charlottesville, VA).

2.5. Immunoprecipitation

The S100 and 20S fractions were directly subject to immunoprecipitation. The P100 pellet from the cell fractionation was resuspended in 1% TX-100 contained in 20 mM Tris HCl pH 7.8 and 140 mM KCl, and centrifuged for 10 min at 14,000 rpm to spin down the insoluble debris. The 60S pellet was resuspended in HEPES (30 mM) buffer, pH 7.8 containing 140 mM KCl. The samples were precleared with rabbit IgG (Jackson Laboratories) and Protein A (GE Healthcare-Amersham Biosciences), then divided into two equal samples and incubated at 4 °C for 2 h with either 5 μg anti-APC Ab (C20 rabbit polyclonal IgG; Santa Cruz Biotechnology Inc.) or APC Ab preincubated with 100 M excess blocking peptide (Santa Cruz Biotechnology Inc) as control followed by 1 h incubation with protein A beads. Beads were pelleted and washed five times with Tris buffer, 50 mM, pH 7.4 with 140 M MnCl2: 0.1% NP-40; 0.1% Deoxycholate; 0.01% SDS or Tris buffer 20 Mm pH 7.5, 140 mM KCl and 0.1% Triton X100.

2.6. Cation exchange chromatography

The 60S pellet was resuspended in isotonic buffer and was applied to a cation exchange Hitrap SP column (GE Healthcare-Amersham Biosciences). Proteins were eluted with 30 mL linear gradient of NaCl in HEPES buffer (10–900 mM), pH 7.2 and analyzed by western blotting for distribution of APC, α and γ-tubulins and IQGAP1.

3. Results

An analysis of distinct cytoplasmic pools of APC from HCT116 human epithelial colon cancer cells, which express wild type APC [35], was carried out as illustrated in Fig. 1. Velocity sedimentation analysis of the cytosolic S100 fraction showed that 30–40% of the APC sedimented around 60S. The
60S pelleted APC eluted as a broad single peak on a sephacryl S100 gel filtration column where bluedextran (~2000 kDa) was eluted (data not shown), which is comparable to the calculated molecular weight of particles sedimenting around 60S. We could not detect any E-cadherin in either the S100 fraction or the 60S pellet, indicating that these fractions were free of membranes (Fig. 2a). Moreover the 60S APC complex failed to float on equilibrium density gradients (Fig. 2a). This ruled out any association of the 60S APC with membrane fragments. Analysis of soluble APC pools from two different epithelial cancer cell lines revealed that axin sedimented around 20S indicating that this pool represents components of the β-catenin destruction complex; with none in the 60S fraction of APC [31]. Nonetheless APC has multiple β-catenin binding sites, and a fraction of β-catenin could potentially be associated with the 60S APC fraction in these cells. However we could not detect any coimmunoprecipitation of β-catenin with APC from the 60S pellet (Fig. 2b) in contrast to the total cytosolic pool of APC or the 20S fraction where β-catenin did communoprecipitate with APC (Fig. 2c and Fig. 9). The absence of β-catenin in the 60S fraction further confirms our earlier report that the 60S APC complex is not likely to represent the β-catenin destruction complex as part of the Wnt signaling pathway. We propose that the 60S APC complex is a distinct cytosolic protein complex with molecular weight of 2000–3000 kDa and does not associate with membranes or the β-catenin destruction complex.

### 3.1. 60S APC is not associated with proteins involved in microtubule plus end stabilization or microtubule transport protein KAP3

We decided to ask whether the 60S APC fraction represents the complex of APC associated with the microtubule plus end binding proteins with possible function in microtubule stabilization and cytoskeletal regulation [26]. The fractionation patterns of both EB1 and diaphanous, which have previously been shown to interact with APC, during velocity sedimentation of the S100 fraction was very distinct from the APC distribution (Fig. 3). EB1 sedimented as a distinct pool around ~5S while diaphanous fractionated around 5–10S. We could not detect either of the proteins in the fractions sedimenting around 60S. Small amounts of both the proteins pelleted during differential centrifugation to enrich the 60S pellet, but neither protein cofractionated when the 60S pellet was refractionated by velocity sedimentation nor communoprecipitated with APC (data not shown). Therefore the 60S APC complex identified in our study does not appear to be a part of the microtubule plus end stabilization complex containing EB1 and diaphanous. We also tested whether 60S APC is a part of the transport complex that could potentially move along the microtubules using the kinesin family of plus-end directed motor proteins through its interaction via KAP3 [23]. The soluble pool of KAP3 sedimented around 10S; with none detectable in the heavy fraction where 60S APC was present (Fig. 3). KAP3 also failed to communoprecipitate with APC from 60S pellet (data not shown). Therefore we do not think that the 60S APC is the APC containing complex that has been reported to move along the microtubule toward the plus end.

### 3.2. Potential interaction of α- and γ-tubulins with the 60S APC complex

APC can interact with microtubules directly through its basic amino acid rich domain present near its carboxy terminal [6], and we therefore asked whether it interacts with tubulin in the 60S complex. α-Tubulin could be detected in all fractions across the velocity gradient with the majority of the tubulin protein fractionating in the lighter fractions around 5S–20S, and small amounts trailing into the region around 60S pool of proteins (Fig. 4a). The sedimentation pattern of both APC and α-tubulin in velocity sizing gradients of the S100 fraction did not change even after the cells were preincubated with microtubule depolymerizing agent nocodazole (33 μM) (data not shown), indicating that the tubulin containing complexes or polymers fractionating around 60S were resistant to drug induced depolymerization. We also asked whether 60S APC interacts with other microtubule associated proteins, like MAP4, the most abundant non-tubulin component of microtubule that has been shown to stabilize microtubules both in vivo and in vitro [36]. However the majority of the MAP4 protein did not copurify with 60S APC, and instead fractionated around 5–10S (Fig. 3). Even though trace amounts of this protein were detectable in fractions sedimenting around 20–
40S, MAP4 failed to coimmunoprecipitate with 60S APC. Since APC has also been reported to localize to centrosomes [37], we decided to analyze the distribution of γ-tubulin, an integral component of the centrosomes, with a proposed role in microtubule minus end nucleation [38]. γ-Tubulin was also distributed broadly across the gradient and enriched around 25–40S (Fig. 4a). This pool likely represents the soluble γ-tubulin ring complex known to sediment around 40S [39]. The distribution profile of γ-tubulin also overlapped with 60S APC fraction across the gradient, although it was not enriched in the peak fractions of 60S APC complex.

In order to determine whether the small amount of either α- or γ- tubulin sedimenting in the 60S fractions is associated with the 60S APC complex, we refractionated the 60S pellet on the sucrose velocity gradients as described earlier (Fig. 4b). As expected 90% of the APC sedimented around 60S, confirming that pelleted APC from the soluble pool in our high speed centrifugation step largely comprised of the 60S APC. The majority of the α and γ- tubulin enriched in the 60S pellet cosedimented with APC. To assess whether either form of tubulin present in the 60S pellet copurifies with APC as a single complex, proteins from the 60S pellet were fractionated by cation exchange chromatography (Fig. 5). Most of the APC protein from 60S pellet eluted as a tight peak at salt concentration of 330–430 mM (fractions 9–11). α-Tubulin was distributed as a broad peak at salt concentrations ranging from 250 mM to 550 mM (fractions 7–14) with peak fractions overlapping with the APC peak fractions, while γ-tubulin exhibited a very broad elution profile at salt concentrations from 250 mM to 750 mM (fractions 7–17) which overlapped with the APC and α-tubulin profile. Therefore, although the α and γ-tubulins in the 60S fraction did not entirely copurify with APC, it is possible that the peak fractions represent a complex with APC.

To directly test whether either form of tubulin physically associate with APC in the 60S fraction, the 60S pellet was subjected to coimmunoprecipitation analysis. Minor fractions of the 60S pellet α- and γ- tubulins coimmunoprecipitated with APC from this pool (Fig. 4c). Even though majority of the tubulin proteins from the 60S pellet did not coimmunoprecipitate with APC, the small fractions associating with APC appeared to be specific because tubulin did not coimmunoprecipitate in controls preincubated with 100 M excess of specific antibody blocking peptide. Furthermore we
did not observe any association of other known binding partners of APC in our immunoprecipitation analysis, indicating that there were no general nonspecific interactions detected by coimmunoprecipitation. Also because APC is not associated with most of the tubulin species separated by copurification and coimmunoprecipitation, it is clear that APC does not nonselectively bind to all tubulin polymers in the cells. We could not determine the stoichiometry of APC and tubulin interaction since the detection method and the sensitivity of the antibodies used widely differed. However considering that the endogenous APC levels are extremely low in the cells compared to α-tubulin, which is an abundant molecule, and that the endogenous APC has been shown to colocalize only with a subset of microtubules in the cells [6], we think that the small amount of tubulin associating with the 60S APC is quite significant. This raises the possibility that this APC complex has some novel function in cytoskeletal regulation in epithelial cells apart from its proposed role in microtubule plus end stabilization.

3.3. IQGAP1 is a potential component of the 60S APC complex

IQGAP1, an effector of Rac1 and CDC42, has been shown to interact with APC [28]. Activated Rac1/CDC42 form a ternary complex with APC and IQGAP1 in migrating Vero cells. It has been hypothesized that this complex could potentially link actin cytoskeletal network and microtubule dynamics during cell polarization and directional migration. IQGAP1 from the S100 fraction distributed broadly across the sucrose gradient, with most of the protein sedimenting around 5–20S (Fig. 6a). However, we could detect small amounts of IQGAP1 in fractions where 60S APC complex sedimented. When the 60S pellet was refractionated by velocity sedimentation, a fraction of IQGAP1 enriched in the same fraction where most of the APC sedimented (Fig. 6b). IQGAP1 from the 60S pellet eluted from the ion exchange column at salt concentrations of 400–500 mM. The peak was separated from the peak of APC (Fig. 5), which suggests that most of the IQGAP1 in 60S pellet is not directly associated with APC. However a small fraction of the 60S pelleted IQGAP1 was also coimmunoprecipitated with APC from this pellet compared to control preincubated with 100 M excess antibody specific blocking peptide (Fig. 6c). Actin from S100 fractions sedimented around 5–10S and was not detectable in the 60S fraction. Our data suggest that a fraction of IQGAP1 specifically associates with APC in the 60S complex, even though most of the IQGAP1 did not copurify with APC in our analysis.

3.4. Interaction of APC with putative binding partners in different cell fractions.

Because we did not observe any of the reported interactions of EB1, KAP3, and mDia with APC in the 60S complex, we examined whether these interactions might occur in different subcellular fractions. Of particular interest is the P100 fraction containing membranes and membrane-cytoskeletal elements, since some of the microtubule-associated proteins are proposed to interact with APC at or near the cell cortex [23, 26]. EB1, KAP3, mDia, and MAP4 were detectable in the P100 (P) fraction (Figs. 7 and 8), but at extremely low levels compared to their amounts in the cytosolic fraction (S100, S). Note that virtually all the E-cadherin fractionated in the P100 fraction, demonstrating complete pelleting of plasma membranes. Such low amounts of microtubule-associated proteins in the P100 fraction could be due either to minor contamination with cytosolic proteins or to very small amounts present in the membrane-cortex. Nonetheless, the proportions of these proteins present in the P100 fraction relative to the S100 is very much less than the proportion of APC in the P100 (≈ half), indicating that significant amounts are not stably associated with membrane-cortical associated APC. We did not detect any EB1, KAP3, mDia, or MAP4 in immunoprecipitates of APC solubilized from the P100 fraction (Fig. 7, IP), but this is not surprising given how little of these proteins were present in the P100 to begin with.

Greater amounts of IQGAP1, α-tubulin, and γ-tubulin were present in the P100 fraction, presumably due either to their
membrane association or to their presence in large cytoskeletal elements, or both. We attempted to determine whether these proteins in the P100 co-immunoprecipitate with APC. As reported previously [31], it is difficult to solubilize APC from the P100 membrane fraction (compare lanes I and S2 in Fig. 7). We did not detect IQGAP1, α-tubulin, or γ-tubulin in immunoprecipitates of APC solubilized from the P100 fraction. Of course, this does not exclude the possibility that any of the tubulin or microtubule-associated proteins interact with APC in the insoluble fraction, but they clearly do not remain associated after detergent solubilization.

We also examined whether the tubulins and microtubule-associated proteins associate with APC in the total cytosolic (S100) fraction, which includes both the 60S and 20S APC complexes (Fig. 8). As observed for the 60S complex, small amounts of IQGAP1, α-tubulin, and γ-tubulin specifically co-immunoprecipitate with APC (IP) and their immunoprecipitation is inhibited by a specific antibody blocking peptide (B.P.). In contrast to what is observed for the 60S complex, β-catenin specifically co-immunoprecipitates with APC in the S100 fraction, presumably due to their interactions in the 20S APC complex [31]. We did not detect any mDia, KAP3, MAP4, or EB1 in APC immunoprecipitates from the S100 fraction (Fig. 8; IP).

Since we observed an interaction of APC with IQGAP1, α-tubulin, and γ-tubulin in both the 60S fraction and the total cytosolic fraction S100, we decided to ask whether these interactions exist in the 20S APC fraction. Small amounts of IQGAP1, α-tubulin, and γ-tubulin specifically coimmunoprecipitated with APC in the 20S fraction (Fig. 9), in contrast to 60S APC (Fig. 2b).

4. Discussion

Confluent HCT116 cells have two distinct soluble pools of APC of size 20S and 60S [31]. The 20S APC fraction is associated with axin, but we could not detect any of the components of β-catenin destruction complex in the 60S APC fraction. However APC can potentially bind to β-catenin through its multiple binding sites. The absence of β-catenin in the APC coimmunoprecipitate from the 60S complex indicates that it either has a very low affinity for the form of APC in this fraction, or else its binding sites are sheltered by other protein interactions. β-catenin in these cells is capable of interacting with APC since it coimmunoprecipitated with APC in the total cytosolic fraction S100 or the 20S fraction (Figs. 8 and 9). APC in the 60S fraction was not associated with any membranes either. Therefore we propose that the 60S fraction of APC identified in confluent HCT116 cells is a discrete soluble high molecular weight protein complex that is distinct from the complex regulating β-catenin destruction.

APC is also known to have a role in microtubule function [6]. Because α-tubulin specifically coimmunoprecipitates with APC in the 60S fraction, the 60S APC complex may have a role in microtubule function or dynamics. We do not believe that the small fraction of the α-tubulin in our immunoprecipitation analysis was due to some nonspecific interaction occurring after cell lysis, because we did not see nonspecific association of APC with other known binding partners like EB1, KAP3, β-catenin etc. The fact that the APC fractionates differently
from the bulk of the α-tubulin in the velocity gradient demonstrates that the 60S APC complex does not simply associate with all microtubules or tubulin polymers in the cell. Our observation is in concordance with the previously reported colocalization studies where endogenous APC was shown to bind to only a subset of microtubules [6]. The association of α-tubulin with the 60S APC complex raised the possibility that it represents a microtubule plus end associating complex. APC has been proposed to play a role in microtubule plus end stabilization in migrating cells [26] through its association with EB1 and mDia. It has also been reported that APC can utilize kinesin family of motor proteins KIF3A–KIF3B via its interaction with KAP3 to move along the microtubule toward the growing plus end [23]. However none of these microtubule plus end binding partners of APC, EB1, diaphanous (mDia) or the kinesin associated protein KAP3 could be found associated with the 60S APC complex. The absence of these interactions suggests that the 60S APC is unlikely to be the APC complex involved in microtubule plus end stabilization [26], and the absence of KAP3 suggests that 60S APC is not simply derived from the complex being transported along the microtubules toward the plus end tips.

The reported interactions of APC with the microtubule binding proteins such as EB1, KAP3 and mDia were not observed in either the total cytosolic fraction S100 or the solubilized membrane/dense fraction P100 in highly confluent HCT116 cells. These findings for EB1 are consistent with previous publications, in which co-immunoprecipitation with APC has not been observed despite other evidence for interactions [24,44]. Similarly, the published co-immunoprecipitation of mDia was barely detectable, despite the other evidence for interactions [26]. KAP3 has been reported to co-immunoprecipitate with APC in MDCK cells [23]. The reason we did not observe the KAP3 co-immunoprecipitation with APC in HCT116 cells is not clear. However, a possible explanation is that the interactions of some of these proteins with APC may be very transient and only occur during cell polarization in migrating cells [23,26,44], whereas our studies were all performed with confluent epithelial monolayers, a state of cell growth needed for the formation of the 60S complex in epithelial cells [31].

It is important to note that we also found γ-tubulin associating with APC in the 60S fraction. APC has been previously shown to colocalize with γ-tubulin near the nucleus in MDCK cells, where APC preferentially localized to one of the two centrosomes [37]. Even though the 60S fraction of proteins isolated in our experiments after differential centrifugation could not contain intact centrosomes, large amounts of γ-tubulin exist in the soluble pool in mammalian cells as two discrete complexes [39], a large γ-tubulin ring complex (γ-TuRC, 25–32S) and a small γ-tubulin small complex (γ-TuSC, 6–9.8S) [41]. γ-TuRC can interact with a subset of well-formed microtubule minus ends or with tubulin subunits. The ring complex has an approximate molecular weight 2000 kDa and contains eight proteins in addition to 10–13 γ-tubulin molecules per complex [42]. Most of the γ-tubulin distributed around 25–40S during the velocity sizing analysis in our study is probably part of this ring complex. Even though 60S APC does not copurify with this ring complex, it is possible that the 60S APC complex could represent an association of APC and other proteins with the ring complex to give rise to a larger complex. A variety of
genetic and biochemical experiments have shown that γ-tubulin is required for microtubule nucleation [38] and half of the microtubules formed in the presence of γ-TuRC in vitro appears to have one complex at their minus end [43]. Considering that the 60S APC complex is present at very low levels in these cells and interacts with a selective fraction of α and γ-tubulin, it is tempting to speculate that this discrete fraction of APC play a role in microtubule nucleation at the minus end.

Recently APC has also been shown to interact with IQGAP1, a protein involved in cytoskeletal organization [28]. Activated Rac1 and Cdc42 can recruit APC/IQGAP1 and it has been proposed that IQGAP1 links APC to actin filaments for cell polarization and directional migration. The presence of IQGAP1 in the 60S APC fraction raises the possibility that this distinct fraction of APC has the potential to interact with actin cytoskeletal network through its association with IQGAP1. It seemed that only a small fraction of the total IQGAP1 copurified with APC. Because of the difficulties in calculating the stoichiometry of APC–IQGAP1 interaction due to the differences in antibody sensitivity and detection methods, it is difficult to say whether this protein is a major binding partner of 60S APC complex. Moreover, no actin is present in the 60S APC fraction. However it remains possible that this 60S APC represents a complex that links microtubule with actin network through IQGAP1 as previously described [28].

It is also important to note that the interactions between APC and IQGAP1, α-tubulin and γ-tubulin were also observed in 20S fraction (Fig. 9). This could imply that the 60S APC complex is formed from the 20S complex plus additional proteins. However, the interaction between β-catenin and APC was only observed in the 20S fraction but not in the 60S complex (Figs. 2b and 9), indicating that they are probably distinct protein complexes and may have different roles in the cell.

Our data suggest that APC in the 60S complex identified in HCT116 cells is a discrete high molecular weight protein complex. This distinct APC complex could be a pool of APC that plays a role in cytoskeletal regulation or it may have some novel function in the cell yet to be identified.

Fig. 8. Analysis of APC interaction with potential binding partners in the cytosolic fraction (S100). Confluent HCT116 cells were lysed and fractionated as described above. The distributions of APC and potential interaction partners in the cytosolic fraction (S100) and the membrane fraction (P100) were analyzed by western blot (TL: Total Lysate, PNF: Post Nuclear Fraction, S: S100, P: P100). The total cytosolic fraction (S100) was subject to immunoprecipitation with anti-APC Ab or APC Ab preincubated with 100M excess blocking peptide. Immunoprecipitates were analyzed by western blotting (IgG: Rabbit IgG Control, IP: APC antibody (C-20) immunoprecipitate, B.P.: immunoprecipitation with blocking peptide, sn: supernatant after immunoprecipitation). Note that the IP and B.P. samples shown are 5X amount comparing to the supernatant (sn).

Fig. 9. Analysis of APC interaction with potential binding partners in the 20S fraction. Confluent HCT116 cells were lysed and fractionated as described above. The 20S fraction was subject to immunoprecipitation with anti-APC Ab or APC Ab preincubated with 100 M excess blocking peptide. Immunoprecipitates were analyzed by western blotting (IgG: Rabbit IgG Control, IP: APC antibody (C-20) immunoprecipitate, B.P.: immunoprecipitation with blocking peptide, sn: supernatant after immunoprecipitation). In the APC blot, the IP and B.P. samples were loaded in equal proportions to the supernatant (sn) samples in order to show the efficiency of immunoprecipitation; in the other blots the IP and B.P. samples were loaded at 10 times the proportion of the supernatant (sn) samples to better detect the small amounts of co-immunoprecipitating proteins.
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Appendix A. Supplementary data


References


