Interaction between Ku80 protein and a widely used antibody to adenomatous polyposis coli

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Adenomatous polyposis coli (APC) is a large multifunctional tumour-suppressor protein. It is central to many processes including developmental regulation and it is mutated in most cases of sporadic colon cancer. Germline mutations in APC lead to the condition known as familial adenomatous polyposis (FAP), which predisposes the affected individuals to colorectal cancer. Furthermore, mutations in APC are found in the majority of sporadic cases of colon cancer. There have been many published studies concerning the cellular localisation of APC, this being fundamental to our understanding of its function, but there has also been much concern over the specificity of certain commercially available antibodies to APC. Here we report that the widely used antibody APC(N15) demonstrates a strong interaction with the Ku80 subunit of the Ku heterodimer under defined experimental conditions. Based on the data presented here, we suggest that APC(N15) is not suitable for many applications used for the study of APC.

MATERIALS AND METHODS

Cell lines
SW480 cells (ECACC) were grown in DMEM/10% FBS, HCT116 cells (ECACC) were grown in McCoy’s 5a medium/10% FBS.

Immunofluorescence
Cells were seeded (5 × 104 cells) in 40 mm tissue culture dishes. Cells were washed in PBS, fixed in 4% paraformaldehyde for 20 min at RT, washed in PBS before being permeabilised in 0.2% Triton X-100 for 15 min at RT. After a further PBS wash, cells were blocked in PBS – 5%FBS for 1 h at RT. Cells were incubated with primary antibodies diluted in PBS – 5%FBS for 30–60 min at 37°C, rinsed in PBS (×3) for 5 min before incubation for 30–60 min at 37°C in secondary antibodies diluted in PBS – 5%FBS. Following secondary antibody incubation, cells were washed (×3) in PBS and stored in PBS for imaging using a Zeiss Axioplan 2 and LSM510 confocal microscope. Antibodies and dilutions are as follows: APC(N15) 1:75 (Santa-Cruz Biotechnology), Ku(p80) 1:50 (Oncogene).

Western blot
Whole-cell lysate was prepared (50 mM Tris-HCl, pH7.4, 100 mM Kac, 0.1% (v/v) Triton, 1 mM AEBSF, 2 µg ml⁻¹ aprotinin, 10 µM bestatin, 10 µM E64, 1 UM pepstatin, 100 µM leupeptin, 1 mM sodium ortho vanadate) and cell extracts resolved by SDS-PAGE. Gels were electrophoretically transferred onto PVDF membrane.

Keywords: Ku80; adenomatous polyposis coli (APC); N15 antibody; crossreactivity
Blots were probed with antibodies diluted as follows: APC(N15) 1:1000 (Santa Cruz Biotechnology, California), Ku80 1:200 (Oncogene, Germany).

**Immunoprecipitation**

Immunoprecipitation was performed essentially as described in Chan *et al.* (2002). The lysis buffer used was the same as for Western blotting above. Silver staining was according to the manufacturer's instructions (Biorad, Hertfordshire, UK).

**Mass spectrometry**

In-gel protein samples were automatically digested using a Micromass MassPREP Station (Micromass, Wythenshawe, UK). MS protein analysis was carried out by Micromass using electrospray MS and MS/MS on a Micromass Q-TOF2 mass spectrometer. All data were processed automatically by means of Protein Lynx software, protein identification was achieved by analysis with ProteinLynx Global Server version 1.0.

**RESULTS**

In an effort to isolate APC and its binding partners, the antibody APC(N15) was incubated with whole-cell lysate from SW480 colon carcinoma cells. SW480 cells contain a truncated version of APC of 150 kDa as a result of a stop codon caused by frameshift mutation. Protein A–agarose beads were used to pull down the antibody–protein complex allowing protein analysis by SDS-PAGE. Following silver staining, two bands of 70 and 80 kDa showed a reproducible enrichment; however, there did not appear to be an equivalent band of the expected size for APC (Figure 1). Both bands were excised, and analysed by mass spectrometry. The samples were unambiguously identified as an ATP-dependent DNA helicase class II 70 kDa subunit (Ku70) and an ATP-dependent DNA helicase class II 80 kDa subunit (Ku80) (Micromass, Wythenshawe, UK). Protein identifications were made by matching both peptide masses and sequences of the digested proteins (data not shown).

Adenomatous polyposis coli has been shown to interact with DNA (Deka *et al.*, 1999), leading to the possibility that interaction with the Ku heterodimer may have occurred indirectly through DNA molecules with double-strand breaks. To eliminate this possibility, the IP was repeated in the presence of DNase. The removal of DNA was confirmed by running a sample on a 1% (w/v) agarose gel before visualising nucleic acid with ethidium bromide (data not shown). In the absence of DNA, Ku70 and Ku80 persistently immunoprecipitated with antibody APC(N15).

A final possibility to account for the coimmunoprecipitation interaction was that one or other of the Ku proteins was interacting nonspecifically with the APC(N15) antibody. To address this, an IP from SW480 whole-cell lysate was performed using an antibody to Ku80. The resulting immunoprecipitate was split into two and resolved by SDS-PAGE, each sample alongside an aliquot of purified DNA–PK complex (which includes Ku70 and Ku80), as positive control. One set was visualised by silver staining, while the other was transferred onto PVDF membrane and immunoblotted first with the Ku80 antibody and then APC(N15). The silver-stained gel revealed two enriched bands of 70 and 80 kDa (data not shown), identical in appearance to those pulled down by APC(N15). The immunoblot demonstrated that both the Ku80 and APC(N15) antibodies detected the Ku80 subunit (Figure 2).

**DISCUSSION**

We present evidence that the widely used APC antibody, APC(N15), demonstrates a strong interaction with the Ku80 subunit component of the Ku heterodimer. This antibody is not suitable for immunoprecipitation of its intended antigen, although it is extremely efficient at affinity purifying the Ku heterodimer. As others have commented (Mogensen *et al.*, 2002), a typical immunoblot with APC(N15) of whole-cell lysate reveals a number of intense bands between 40 and 90 kDa (one of these intense bands is Ku80), but only a weak band of the correct size for APC itself (Figure 3). Furthermore, a band corresponding to the size of full-length APC was observed on longer exposures of SW480 cells, which do not contain full-length APC. This is in accordance with Mogensen *et al.* (2002), who detected a similar band in DLD1 cells that also express a truncated APC protein. This indicates that the full-length band observed is most likely because of a further crossreaction.

There has been some concern as to the suitability of the widely used antibody APC(N15) for immunohistochemistry (Rosin- Arbesfeld *et al.*, 2001; Mogensen *et al.*, 2002), although this has yet to be fully resolved. The observation that APC(N15) does not detect ectopically expressed GFP-tagged APC or microtubule-associated APC (Rosin-Arbesfeld *et al.*, 2001) adds weight to this concern.

We looked at colocalisation of APC(N15) and Ku80 (Figure 4) and observed nuclear staining with both antibodies in SW480 and HCT116 cells. Although staining of nuclei is greater with Ku80...
compared with APC(N15), it is possible that this is because of different titres of antibody. We also noted that the levels of nuclear staining with APC(N15) in SW480 and HCT116 cells are similar in contrast to the staining patterns observed using the APC antiserum raised by Nathke et al. (1996). Finally, although APC(N15) detects additional material outside the nucleus, we cannot exclude the possibility that APC(N15) is detecting other proteins in addition to Ku80 (see immunoblot, Figure 3).

It is noteworthy that two reports published simultaneously, and describing the nuclear localisation of APC in SW480 cells (Henderson, 2000; Rosin-Arbesfeld et al., 2000) presented almost identical images despite one of them (Henderson, 2000) using APC(N15). One explanation could be that this presentation is fortuitous because of the abundance of nuclear APC observed in SW480 cells.

Based on the evidence we have presented here, we would suggest that APC(N15) is unreliable because of crossreactivity and is therefore not suitable for immunodetection of APC.

SUMMARY

The adenomatous polyposis coli (APC) gene and its expressed product are highly studied because of its role as a tumour-suppressor protein. Here we report that the widely used APC antibody (N15) demonstrates a strong interaction with the Ku80 subunit of the Ku heterodimer.

ACKNOWLEDGEMENTS

We thank Micromass (Dr Jonathon Coffey) for undertaking the q-TOF analysis on our behalf. Dr Gwyndaf T Roberts was supported by BBSRC (Grant number 35086). Melanie L Davies was supported on a grant from the Tenovus Cancer Research Charity (Grant number 35141).
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