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# Truncated adenomatous polyposis coli (APC) tumour suppressor protein can undergo tyrosine phosphorylation

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**Abstract**

Numerous mutations in the *adenomatous polyposis coli* (*APC*) gene have been described in colorectal cancer. The vast majority introduce nonsense codons leading to the production of truncated *N*-terminal APC fragments. Mutations occurring before APC codon 158, have been associated with an attenuated form of familial *adenomatous polyposis* whereas those occurring at codon 168 or beyond lead to the characteristic form of the disease. These 10 amino acid residues of APC contain a YYAQ motif which appears to constitute a potential SH2 binding domain similar to a sequence present in tyrosine kinase receptors that activate STAT 3 when phosphorylated. We have expressed a recombinant, *N*-terminal APC fragment in bacterial cells, and shown that it can indeed undergo tyrosine phosphorylation in this domain. We used site-directed mutagenesis to confirm the specificity of the reaction. These observations raise the possibility that tyrosine phosphorylation may be another mechanism involved in controlling APC function. 

*Keywords:* APC; Tyrosine phosphorylation; STAT3; HT-29 cells

## 1. Introduction

Mutations of the *APC* gene are responsible for familial *adenomatous polyposis* (FAP) and are also observed in adenomas at the earliest stages of sporadic colorectal cancer development. APC is known to associate with microtubules [1] as well as β-catenin [2] and plakoglobin [3] which are involved in cell adhesion and Wnt signalling. EB1 [4] and DLG, the human homologue of the *Drosophila* discs large tumour suppressor protein [5], also bind APC. The occurrence of activating β-catenin mutations in colorectal cancers which do not have *APC* mutations suggests that control of β-catenin-mediated transcription is a key element in preventing carcinogenesis [6]. Of the many *APC* mutations which have been reported to date, the vast majority are chain terminating. This leads to the expression of truncated proteins which are then unable to bind to some or all of the proteins discussed above. Essentially all mutations are confined to the 5′-half of *APC* and the majority of somatic mutations (approximately 65%) are found in the 'mutation cluster region' [7].

There is also some indication of an association between mutations which occur at the extreme 5′ end of the gene and attenuated FAP [8]. Mutations which occur up to and including codon 158 of the *APC* gene lead to an attenuated form of FAP [7].

However, mutations which occur at codon 168, or distal to this, apparently lead to the classical form of FAP [7]. Although it might be argued that alternative splicing of mutations in 5′-exons of *APC* might be an explanation for attenuation, this does not explain the attenuated picture for mutations between codons 141 and 158, because residues 141–177 are all encoded within exon 4 of *APC*. This led us to hypothesise that within the intervening 10 residues between 158 and 168 there may be amino acids which are functionally important and play a role in governing the severity of FAP.

We noted that a potential tyrosine phosphorylation site (-$^{158}$YYAQ-) exists in this region [9]. The site is preceded by a motif which scores positive on Prosite analysis for phosphorylation by tyrosine kinases [10]. The YxxQ sequence is conserved in the gp130 signal

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transducing subunit of the IL-6 receptor where it is essential for activation of the transcription factor, Stat 3 [11]. It is also conserved in the cytoplasmic domains of a number of other receptors [12,13], where again it has been shown to be necessary for Stat3 binding. Furthermore, the cytoplasmic inhibitor of Stat3, PIAS3, which binds to activated Stat3 *in vivo*, also contains the consensus sequence — YxxQ [14].

We therefore attempted to demonstrate phosphorylation at this putative SH2 domain and recognition site for Stat3 in APC [15]. We constructed amino-terminal fragments of the normal *APC* gene and homologous fragments mutated at either or both of the key tyrosine residues (158 or 159) and expressed the corresponding recombinant proteins as in-frame fusions with the FLAG epitope under an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter. We also used these constructs to transform bacterial TKX1 cells, which harbour an inducible, mammalian *tyrosine kinase (TK)* gene [16], and analysed the expressed APC–FLAG proteins for evidence of their ability to undergo tyrosine phosphorylation.

## 2. Materials and methods

### 2.1. Construction of APC–FLAG expression vectors

Full length pAPC-CMV (a gift from Professor B. Vogelstein) was digested with *BamHI* and *KpnI*, to generate a 635 bp 5′-terminal fragment. This was ligated into the corresponding sites of the pUC vector (New England Biolabs, Hitchin, UK). Site-directed mutagenesis was then performed using the USE mutagenesis kit (Pharmacia, Little Chalfont, Bucks, UK) and following the manufacturer's protocol. The following primers were used: d(GGAAAAAGACTGG*TATTTC*GCTCAACTTCAG); d(GGAAAAAGACTGG*TTCTAC*GCTCAACTTCAG); and d(GGAAAAAGACTGG*TTCTTC*GCTCAACTTCAG). Three different reactions were performed, to give individual mutations of the two tyrosine residues, as well as the double mutant. These were confirmed by DNA sequencing in both directions. In all cases, tyrosine (Tyr) was mutated to phenylalanine (Phe). Each of the 212 amino acid *APC* fragments in the pUC vector were then subcloned into a FLAG vector (IBI) which was used to transform *Escherichia coli* DH5α cells, to allow easier detection of proteins expressed from its IPTG-inducible promoter.

### 2.2. Transformation of TKX1 bacterial cells, expression of APC-FLAG proteins and induction of Elk tyrosine kinase

The four APC–FLAG constructs were used separately to transform TKX1 bacterial cells (Stratagene, Amsterdam, The Netherlands). A fresh overnight culture of each transformation reaction was diluted 1:20 with 2×YTG medium (16 g/l tryptone, 10 g/l yeast extract, 10 g/l NaCl) containing ampicillin (50 μg/ml) and tetracycline (12.5 μg/ml). After growth at 37°C for 2 h, the cultures were induced with IPTG (1 mM) and grown at 37°C for a further 2 h. As a control for expression of the APC-FLAG proteins before induction of the *TK* gene, aliquots were harvested and resuspended in 2×SDS-PAGE loading buffer. The remaining cells were harvested by centrifugation at 3000*g* for 10 min and resuspended in TK induction medium (6 g/l $Na_2HPO_4$, 3 g/l $KH_2PO_4$, 1 g/l $NH_4Cl$, 0.5 g/l NaCl, 2 g/l glucose, 0.1 g/l casamino acids, 10 mg/l indoleacrylic acid, 0.1 ml thiamine–HCl (0.5%), with 1 mM $MgSO_4$) containing ampicillin (50 μg/ml), and tetracycline (12.5 μg/ml). Cultures were incubated at 37°C for 2 h, to allow induction of *TK*, then harvested at 3000*g* for 10 min. Pellets were resuspended in 2×SDS-PAGE loading buffer and analysed using SDS-PAGE.

### 2.3. SDS-PAGE analysis and Western blotting of proteins

Aliquots of the four APC–FLAG protein mixtures were separated using a 12% polyacrylamide gel. The proteins were transferred to PVDF membranes (Millipore, Bedford, MA, USA), in Towbin's buffer using a semi-dry blotting system (Hoeffer). Immunoprecipitated protein samples were separated on 4–12% gradient SDS-PAGE.

### 2.4. Analysis of Western blots

PVDF membranes were blocked with I-Block (Tropix, Boston, MA, USA) as recommended by the manufacturer. Membranes were probed with *N*- or *C*-terminal, anti-APC monoclonal antibodies (Oncogene Sciences, Beeston, Notts, UK, FE9 and IE1, respectively), an *N*-terminal anti-Stat3 antibody (Transduction Laboratories, Lexington, KY, USA), or the PY20 anti-phosphotyrosine antibody. In the latter case, a block of 4% fish gelatin (Sigma, Dorset, UK) in 20 mM Hepes, 135 mM NaCl was used. Secondary antibodies conjugated to alkaline phosphatase (Dako, Ely, Cambridgeshire, UK) were used at a dilution of 1 in 1000 if NBT/X-phosphate, alkaline phosphatase substrate (Boehringer Mannheim, Lewes, East Sussex, UK) was used, or at 1 in 5000 if the Western-Light Chemiluminescence detection system (Tropix) was used.

### 2.5. Characterisation of N-terminal APC fragments in HT-29 cells

HT-29 cell lysates (see below) were subjected to SDS-PAGE and Western blotting without immunoprecipitation, as described above. Blots were probed with the FE9 and

IE1 antibodies. Cycle sequencing (Promega, Southampton, UK) with α-[$^{32}$P]-dCTP (Amersham, Little Chalfont, Bucks, UK) using the *APC* primers dGGAAAATGACAATGGGAATGAA (starts at nucleotide 4628 in the exon 15 open reading frame (ORF)) and the complement of dTACAAACTTCTACCATCACA in the reverse direction (residues 4884 to 4903 in the exon 15 ORF) was performed on genomic DNA from HT-29 cells. HT-29 DNA was also polymerase chain reaction (PCR) amplified within exon 15 using a T7 promoter-containing forward primer (64 bp) with an in-frame start codon (italicised) before the sequence encoding amino acids 654–661 of APC (dGACTAATACGACTCACTATAGGAACAGACCACC***ATG***GCTCAAATCCTAAGAGAGAACAACTGTC) and a reverse primer complementary to dCGTGTGAAAAAGATAATGGACCAG, encoding APC amino acids 1743–1750. The Expand Long Template system (Boehringer) was used to avoid amplification errors. Positive control amplifications of normal *APC* cDNA and normal human DNA were performed in parallel. The 3.3 kb PCR products were purified on agarose gels and isolated by QIAquick extraction (Quiagen, Crawley, UK). PCR products were added to a T7-coupled protein translation reaction (Promega) in a volume of 10 μl containing 10 μCi of $^{35}$S-ProMix (Amersham). Incubation was for 2 h at 30°C followed by separation of $^{35}$S-labelled protein products by 6–12% gradient Laemmli SDS-PAGE.

### 2.6. Immunoprecipitation of APC fragments or Stat3 from HT-29 cells

HT-29 cells maintained in medium containing 3.3 nM EGF (R + D Systems), were lysed 10–14 days after confluency had been reached, in 0.135 M NaCl and 50 mM Tris–HCl (pH 8.0) containing leupeptin (10 μM), pepstatin (3 μM), phosphoramidon (20 μM), PMSF (100 μM), pefabloc (200 μM) and sodium orthovanadate (1 mM). Cell lysates were passed through a 26 gauge needle, three times before centrifugation at 16 000 g for 20 min. The cytoplasmic fraction was precleared with secondary antibody reagent, on magnetic beads. Primary immunoprecipitating *N*- and *C*-terminal anti-APC antibodies were the mouse monoclonals, CF11 and DB1 (Oncogene Sciences) respectively. Immunoprecipitating Stat3 antibody was a rabbit polyclonal to the *C*-terminus (Santa Cruz). The immune complexes formed by the anti-APC antibodies were collected on sheep anti-mouse IgG Dynabeads (Dynal, Wirral, UK). The immune complexes formed with Stat3 antibodies were incubated with biotinylated anti-rabbit immunoglobulins (Dako) followed by M-280 Streptavidin Dynabeads (Dynal). After overnight incubation at 4°C, immune complexes on the magnetic beads were washed in Hepes-buffered saline (HBS) and 0.5% bovine serum albumin (BSA) with PMSF (100 μM) and sodium orthovanadate (1 mM) three times, then twice in HBS. Beads were heated at 95°C in 2×SDS-PAGE loading buffer before SDS-PAGE analysis.

| | |
|---|---|
| Stat 3 recognition motif: | ***YXXQ** |
| Wild type APC: | W**YYAQ**LQNLTKR |
| Mutant 1: | W**YFAQ**LQNLTKR |
| Mutant 2: | W**FYAQ**LQNLTKR |
| Mutant 3: | W**FFAQ**LQNLTKR |

Fig. 1. Amino acid sequence of APC, residues 157–168 and the mutant fragments used to analyse the putative tyrosine phosphorylation site (residues 158 and 159). The recognition motif for Stat3 is shown, with the phosphotyrosine residue involved in Stat3 binding marked *Y, tyrosine.

## 3. Results

### 3.1. APC mutagenesis and cloning into the FLAG vector

In total, four amino-terminal fragments of *APC*, residues 1–212, were constructed. Three of these were mutated: (1) Tyr 158 mutated to Phe; (2) Tyr159 mutated to Phe; and (3) Tyr158 and Tyr159 both mutated to Phe.Phe. The sequences involved between APC residues 157 and 168 and the consensus TK motif are illustrated in Fig. 1. Each of the three mutant sequences and the wild-type *APC* fragment, were individually subcloned into the FLAG vector, such that expressed proteins were in frame with a *C*-terminal FLAG, 8 amino acid, epitope. The four APC–FLAG constructs were used separately to transform *E. coli* DH5α cells. Expression of each construct induced using IPTG was demonstrated by Western blotting followed by probing with the MS2, anti-FLAG, antibody (Fig. 2). A protein of approximately 28.7 kDa was expressed from each construct, as judged by comparison with Novex Sea Blue size markers.


Fig. 2. Protein expression from the four APC–FLAG constructs in *E. coli* DH5α. Lanes 1, 2 and 3, wild-type APC–FLAG; lanes 4, 5 and 6, mutant 1; lanes 7, 8 and 9, mutant 2; and lanes 10, 11 and 12, mutant 3. For each construct: pre-induced sample (lanes 1, 4, 7 and 10); IPTG for 2 h (lanes 2, 5, 8 and 11); IPTG for 4 h (lanes 3, 6, 9 and 12).

### 3.2. Expression and tyrosine phosphorylation of APC–FLAG proteins

The four *APC–FLAG* constructs were then used to transform TKX1 bacterial cells. APC–FLAG protein expression was again induced, *in situ*, using IPTG. Tyrosine phosphorylation was then analysed after expression of the inducible mammalian Elk tyrosine kinase gene in the TKXI cells. APC–FLAG proteins, expressed in these bacterial cells and exposed to the TK were blotted and located using the MS2, anti-FLAG antibody. Again, all four constructs were found to express a 28.7 kDa protein (Fig. 3a). When the blotted proteins were then detected using the PY20, anti-phosphotyrosine antibody, phosphotyrosine was detected in the wild type, *N*-terminal APC protein fragment only (Fig. 3b). Negligible background levels of phosphorylation were achieved with the three mutant proteins. Wild type APC–FLAG protein did not display any phosphotyrosine immunoreactivity when Elk TK expression was not induced (Fig. 4). This demonstrates that *N*-terminal APC–FLAG is selectively phosphorylated on Tyr 158 by the Elk kinase in this system. We cannot totally exclude the possibility that wild-type Tyr159 is also additionally or exclusively phosphorylated, although this does not occur when Tyr158 is absent in the $^{158}F^{159}Y$ mutant. This implies that both tyrosine residues are necessary for the reaction. The possibility that this particular TK is non-specific when highly overexpressed in bacteria is not borne out by the fact that none of the other four tyrosine residues present in the *N*-terminal 212 amino acid fragment (residues 6, 96, 133 and 191) undergoes phosphorylation in any of the three mutant proteins.

### 3.3. Characterisation of N-terminal APC fragments in HT-29 cell

Western blotting of HT-29 cell lysates using the *N*-terminal anti-APC antibody, FE9, detected proteins of approximately 175 and 100 kDa (Fig. 5a). No full length (310 kDa) APC was detectable as bands at the top of the gel. As expected, the *C*-terminal anti-APC antibody IE1 did not detect these truncated proteins in HT-29 cell lysates (data not shown). Sequencing of HT-29 cell DNA showed an extra A residue was inserted between codons 1555 and 1556 producing a stop codon at 1558 to encode a truncated protein of 171 kDa (Fig. 5b). A protein truncation test was performed to assess the region of *APC* in which mutations generating truncated proteins of 175 and 100 kDa would be expected to occur (the 1096 residues from 654 to 1750 were studied as a 121 kDa *in vitro* translation product). This full length product was generated from the normal *APC* cDNA and normal human genomic DNA controls (Fig. 5c, lanes 1 and 3). The codon 1555 mutation (above) in HT-29 cell DNA results in truncation of the 121 kDa *in vitro* translation product to 99 kDa (Fig. 5c, lane 2), corresponding to the 904 residues 654–1558. A second major *in vitro* translation product from HT-29 cell DNA, of approximately 30 kDa is generated (Fig. 5c, lane 2). This implies that a stop codon is present in a second *APC* allele in HT-29 cells at approximately codon 900 (generating a fragment of some 250 amino acids in the protein truncation test). This would be consistent with the 100 kDa APC species detected by Western blotting with the FE9 antibody. We have failed to identify a mutation in this region in HT-29 cell DNA (data not shown). This may imply that abnormal splicing is responsible in some way for generation of the 99 kDa species. All residues in normal *APC* beyond codon 652 are encoded by exon 15.

### 3.4. Immunoprecipitation of APC fragments from HT-29 cells

A monoclonal antibody to the *N*-terminus of APC (CF11) or polyclonal antibody to the *C*-terminus of Stat3 were used to immunoprecipitate extracts from epidermal growth factor (EGF)-stimulated HT-29 cells for SDS-PAGE and Western blotting. Fig. 6(a), lane 1, shows a control experiment where a blotting antibody to the *N*-terminus of Stat3 was used to detect material immunoprecipitated with the *C*-terminal Stat3 antibody. Fig. 6(a), lane 2, shows use of the same Stat3 blotting antibody to detect material immunoprecipitated with

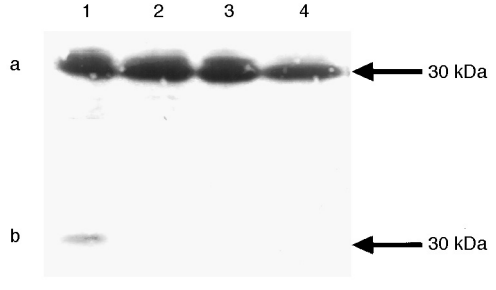


Fig. 3. Phosphorylation of APC fragments in TKX1 bacterial cells by inducible Elk TK. (a) Western blot probed with anti-FLAG antibody: lane 1, wild type APC fragment; lane 2, mutant 1; lane 3, mutant 2, lane 4, mutant 3. (b) Western blot probed with PY20 (anti-phosphotyrosine) antibody: lane 1-wild-type protein; lane 2, mutant 1; lane 3, mutant 2; lane 4, mutant 3.


Fig. 4. Tyrosine phosphorylation in TKX1 bacterial cells requires Elk TK induction. Western blot probed with PY20 antibody. Pre-induced cells with the wild type, *N*-terminal APC–FLAG construct, without IPTG (lane 1); induced with IPTG (lane 2); induced with IPTG and incubated to induce Elk TK expression (lane 3).

the CF11 anti-APC antibody. It appears that some Stat3 is indeed associated with APC in these cells. Fig. 6(b), lane 1 shows a control experiment where *N*-terminal APC fragments precipitated by the CF11 monoclonal are detected with a blotting antibody also to the *N*-terminus of APC (FE9). Two major species of approximately 175 and 100 kDa are detected consistent with the above Western blotting data (Fig. 5a) and in agreement with a previous report describing APC proteins in HT-29 cells [17]. A *C*-terminal anti-APC blotting monoclonal (IE1) did not detect these species, as expected (data not shown). Fig. 6(b), lane 2, shows the corresponding immunoprecipitation using the Stat 3 *C*-terminal antibody, again probed with the blotting antibody to APC *N*-terminus (FE9). The 175 kDa *N*-terminal APC fragment is clearly detected. The 100 kDa *N*-terminal APC fragment does not appear to co-immunoprecipitate so efficiently with Stat3 although the high background makes interpretation difficult. Fig. 6(c) shows the results of immunoprecipitation of HT-29 cell lysates with the Stat3 *C*-terminal antibody and probing with the PY20 anti-phosphotyrosine antibody. Tyrosine-phosphorylated proteins of comparable sizes with the *N*-terminal APC fragments and Stat3 itself were detected.

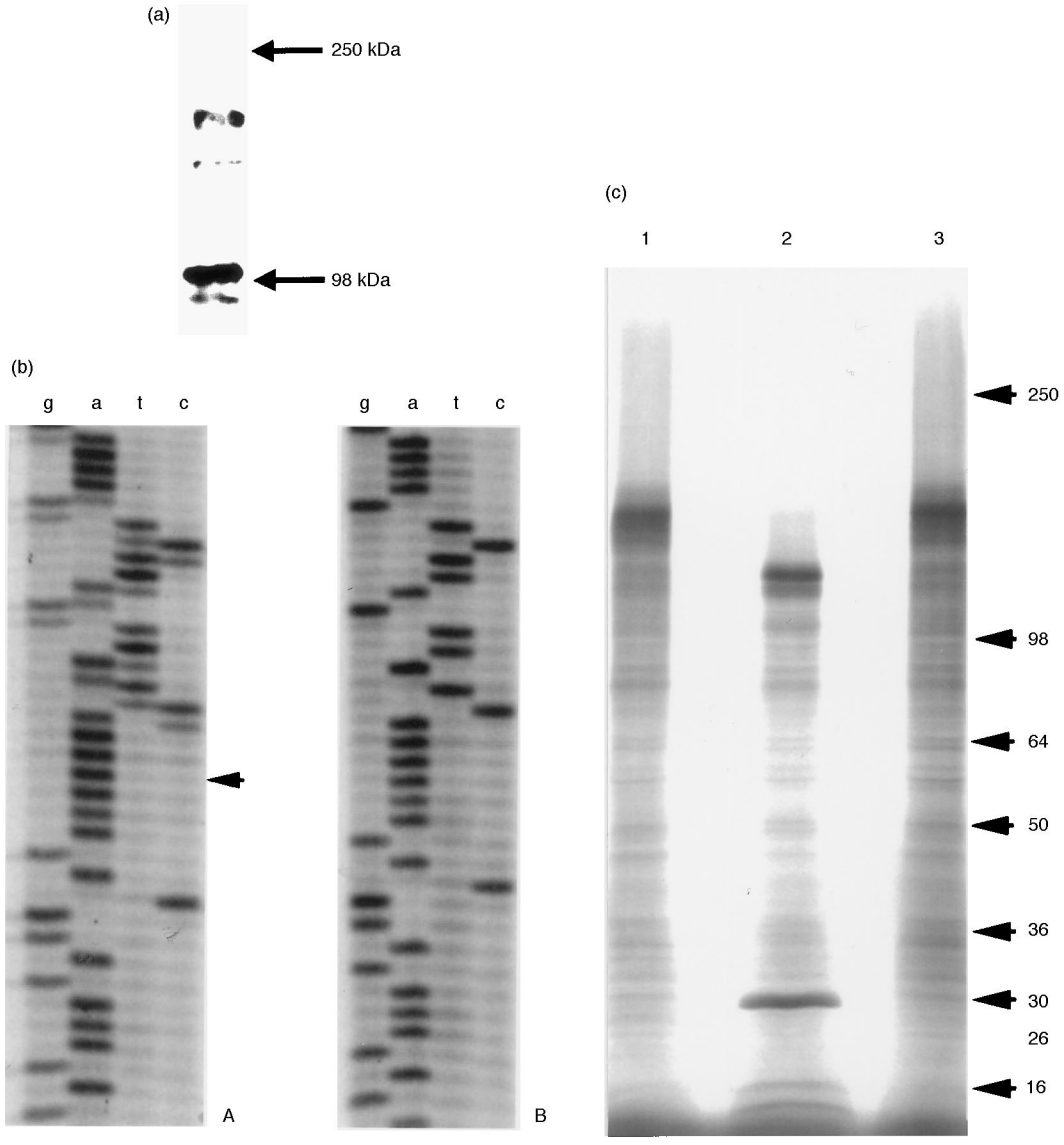


Fig. 5. Characterisation of *N*-terminal APC fragments in HT-29 cells. (a) Western blot of HT-29 cell lysate probed with the *N*-terminal APC antibody FE9. (b) Cycle sequencing of HT-29 cell DNA (A) and normal DNA (B). The run of six A residues into which a seventh is inserted at codon 1555 in HT-29 DNA is arrowed. (c) Protein Truncation Test for APC from HT-29 cells. Lane 1, product from amplified normal APC cDNA; lane 2, product from amplified exon 15 of HT-29 genomic DNA; lane 3, product from amplified normal human genomic DNA. Size markers, Novex See Blue.

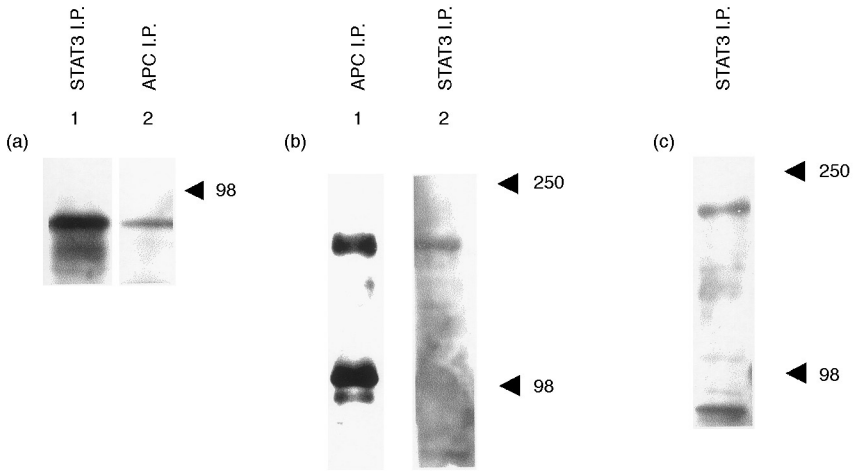


Fig. 6. Co-immunoprecipitation of APC fragments and Stat3 from HT-29 cells. (a) Using *C*-terminal anti-Stat3 antibody for immunoprecipitation and *N*-terminal Stat3 blotting antibody (lane 1); anti-APC monoclonal antibody (CF11) for immunoprecipitation and *N*-terminal Stat3 blotting antibody (lane 2). (b) Immunoprecipitation using CF11 and detection using *N*-terminal anti-APC monoclonal blotting antibody FE9 (lane 1); immunoprecipitation using *C*-terminal anti-Stat3 antibody and detection using FE9 (lane 2). (c) Immunoprecipitation using *C*-terminal anti-Stat3 antibody and detection using PY20 anti-phosphotyrosine antibody.

## 4. Discussion

In this report, we have provided evidence that a tyrosine residue(s), located at position 158 (or possibly 159) of the APC protein, is an accessible phosphorylation site. Expression of a wild-type, *N*-terminal fragment of APC (residues 1–212), in TKX1 bacterial cells, resulted in a tyrosine-phosphorylated protein, detectable using either an anti-phosphotyrosine antibody or an antibody to the FLAG epitope introduced into this recombinant protein. In contrast, if either tyrosine residue 158 or 159 (or both) was mutated to a phenylalanine residue, phosphorylation was very much reduced. It is likely, therefore, that both tyrosine residues are required at this site to allow efficient phosphorylation of APC. We believe this is the first demonstration that APC may be a substrate for a tyrosine kinase(s). Although we have not demonstrated that full length APC is similarly tyrosine phosphorylated in normal cells, the evidence generated using these *N*-terminal fragments now justifies such investigations. Whilst it might be argued that phosphorylation of this recombinant protein in bacterial cells by the induced Elk kinase might be artefactual, this must be regarded as unlikely since negligible phosphorylation was observed of the two proteins which retain a single tyrosine residue at 158 or 159. Furthermore, there is no detectable phosphorylation of the four other tyrosine residues present at amino acids 6, 96, 133 and 191 in all four recombinant proteins.

The evidence that APC fragments have a dominant negative role in tumorigenesis is not compelling given that transgenic mice overexpressing a mutant *Apc* 716 allele on a wild type *Apc* $^{+/+}$ background do not demonstrate the *Min* (FAP) phenotype [18]. Nevertheless, it is intriguing that the absence of residues 158–168 in APC fragments is associated with an attenuated clinical phenotype and perhaps this motif only exerts a detrimental effect in the absence of the wild-type protein. It has been suggested that APC may undergo *N*-terminal dimerisation [19]. It remains to be established how codon 158/159 phosphorylation might affect this process. Tyrosine phosphorylation is a very important mechanism for the control of protein activities [10]. It can produce a binding site for SH2 domains of interacting proteins, specificity arising from the surrounding sequence [15]. Although there are several tyrosines in APC, only those at residues 158 and 159 are preceded by a sequence pattern which scores on a Prosite motif search for phosphorylation by Src kinase family members. The motif is conserved in vertebrate APCs (human, mouse and *Xenopus*) but not in the *Drosophila* or *C. elegans* APC homologues.

The Elk tyrosine kinase which phosphorylated the wild-type *N*-terminal APC fragment was actually the cytoplasmic domain of this mammalian, membrane-bound receptor kinase [20]. The TKX1 bacterial cells used are transformed with a plasmid expressing a 45 kDa kinase domain which is capable of autoactivation after it is induced. The receptor itself is a member of the Eph family whose normal substrates, like the Src-kinases, are other cell surface receptors, often of the same family. Eph receptor signalling overrides cell adhesion and is involved in guiding the migration of neural crest cells. EphA2 (or Eck), a close relative of Elk is found in epithelial cells [16]. The fact that a member of the Eph tyrosine kinase family can phosphorylate an *N*-terminal APC fragment is intriguing. It will be interesting to determine whether the epithelial kinase Eck, or indeed other receptor TKs are also capable of phosphorylating

wild-type APC. APC is known to be phosphorylated at serine and threonine residues [21]. It is a substrate for protein kinase A (PKA), GSK-3β [22] and $p34^{cdc2}$ [23], which implicates APC in the cell cycle. GSK-3β-phosphorylated APC is likely to be involved in the down-regulation of β-catenin, by binding to β-catenin and then targeting it for destruction by the proteosome–ubiquitin pathway [24].

The sequence motif YxxQ (Fig. 1) is also present in the gp130 signal transducer subunit of the IL-6 receptor. This sequence is absolutely required for activation of the transcription factor Stat3 [9,11]. The consensus sequence is also present in the cytoplasmic domains of the IFNAR1 subunit of the Type I interferon receptor [12] and the IL-10 receptor where again it is essential for Stat3 binding [13]. Moreover, PIAS3, which binds to and inhibits activated Stat3 *in vivo*, contains the same motif [14]. The dimerised EGF receptor activates Stat3 directly by tyrosine phosphorylation whereas the gp130 subunit of the IL-6 receptor requires bound Jak1 or Jak2 to phosphorylate Stat3 [25,26]. Stat3 is itself activated by tyrosine phosphorylation which enables it to homodimerise *via* its SH2 domains and be transported to the nucleus. There it binds to palindromic sequences related to γ-interferon activation sites, in a number of promoters [27].

It appears that a truncated APC from EGF-stimulated HT-29 cells (the 175 kDa species which arises from a terminating mutation at codon 1558) may be capable of associating with Stat3. It is possible that truncated APC from the HT-29 cells and Stat3 are able to associate only after the Tyr158 phosphorylation of this APC fragment, although this remains to be demonstrated. Whilst the 212 residue recombinant fragments may not behave exactly like the 1558 residue HT-29 cell APC fragment, there is no reason to believe that the Tyr158 site is masked in the larger protein. The 100 kDa APC fragment in HT-29 cells does not appear to associate so efficiently with Stat3. Analysis of HT-29 cell lysates, using an antibody raised to APC sequence encoded in exon 3, showed that the 175 kDa species of APC is far more immunoreactive than the 100 kDa species, for which a very weak signal was detected (data not shown). It is therefore possible that exon 3 is altered in transcripts which encode the 100 kDa protein. Transcripts of APC which lack exon 3 have been described in HT-29 cells [28] and these also lack exons 2 and 4. It is therefore possible that exon 3 may be absent from the 100 kDa APC fragment and that exon 4, which harbours the putative Tyr 158 phosphorylation site, may be absent also. This could explain why the smaller APC species from HT-29 cells does not apparently associate so effectively with Stat3.

We chose to analyse tyrosine phosphorylation of truncated APC and search for species interacting with it in two convenient experimental systems (TKX1 bacterial cells and HT-29 colorectal cancer cells) because the size of APC (≈310 kDa) and its relatively low levels in many cell lines, make it difficult to study in practice. Moreover, full length APC is absent in colorectal cancers and in this context, the behaviour of *N*-terminal APC protein fragments is particularly interesting. We have now shown that a 212 amino acid, *N*-terminal APC fragment can undergo tyrosine phosphorylation. We have also characterised the truncated 175 kDa APC species in HT-29 cells and shown that this 1558 amino acid fragment may interact with Stat3. We have presented preliminary evidence that the 175 kDa APC fragment may also be tyrosine-phosphorylated when co-immunoprecipitated with Stat3. We now plan to study cells expressing Stat3 and full length APC, to establish whether this interaction can be substantiated in other cell types, whether the Y158 Y159 residues are essential for this, and whether (phosphorylated) APC may suppress the activity of (phosphorylated) Stat3.

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