

The E1 Copper Binding Domain of Full-length Amyloid Precursor Protein Promotes Epithelial to Mesenchymal Transition in DU145 Cells in an Isoform-specific Manner

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ABSTRACT

Epithelial to mesenchymal transition (EMT) confers migratory and dynamic properties on cells and, as such, plays a pivotal role in the development of metastatic, castration resistant prostate cancer. The amyloid precursor protein (APP), although most closely associated with the neurodegenerative condition Alzheimer's disease, has also been linked to the pathogenesis and prognosis of several cancers including prostate cancer.

Aims: To investigate whether over-expression of APP could promote EMT in prostate cancer (PCa) DU145 cells and to determine the molecular prerequisites for this effect.

Methodology: A range of APP molecular constructs were stably expressed in DU145 cells and their effects on EMT were monitored by morphological analysis and by immunoblotting for the EMT marker proteins, E-cadherin and vimentin.

Results: Our results show that the full-length 695 amino acid isoform (APP₆₉₅), but not APP₇₅₁ or APP₇₇₀, promoted EMT via a mechanism requiring an intact extracellular E1 copper binding domain and tyrosine687 within the cytosolic domain of the protein.

Conclusion: Targeting the expression of APP₆₉₅ or the E1 copper binding domain of the protein may, therefore, contribute to therapeutic strategies for the delay or prevention of prostate cancer metastasis.

Keywords: Prostate cancer, epithelial to mesenchymal transition, metastasis, amyloid precursor protein

1. INTRODUCTION

Advanced, androgen-independent, chemo-refractory prostate cancer (PCa) remains incurable. Thus, the identification of new therapeutic targets for the treatment/prevention of metastatic disease would reduce disease mortality.

Epithelial to mesenchymal transition (EMT) is the process by which a polarized epithelial cell assumes a mesenchymal phenotype and facilitates the malignant transformation and metastasis of prostate cancer cells [1-3]. The process begins with a decrease in cellular E-cadherin levels leading to the mechanical disruption of adherens junctions and reduced cell-cell and cell-matrix interactions [4]. E-cadherin is replaced by N-cadherin whilst other proteins such as vimentin and fibronectin replace cytokeratin expression [4]. The repression of proteins that promote EMT might represent a viable therapeutic strategy for the prevention of PCa metastasis.

The amyloid precursor protein (APP) has achieved notoriety for its role in the pathogenesis of the neurodegenerative condition, Alzheimer's disease (AD). Three major isoforms of the protein are expressed in human tissues, APP₆₉₅, APP₇₅₁ and APP₇₇₀ [5]. The main components of the extracellular senile plaques in the AD-afflicted brain are amyloid beta (A β)-peptides which are derived from APP through two sequential proteolytic cleavages by β -secretase (β -site APP-cleaving enzyme 1; BACE1) and the γ -secretase complex [5]. The initial soluble product generated by β -secretase is termed sAPP β (soluble APP beta). In contrast to this 'amyloidogenic' pathway, APP is also processed via a non-amyloidogenic route involving α -secretase cleavage within the A β domain [5]. This latter cleavage precludes A β -peptide formation and generates sAPP α (soluble APP alpha).

More recently, APP has been shown to play a role in cancer. Expression of the protein stimulates colon carcinoma cell proliferation [6] and an increase in APP mRNA expression in oral squamous cell carcinomas is associated with a reduction in patient survival [7]. Thyroid cancers are characterized by up regulation of APP protein and mRNA expression [8] with the former also being enhanced in pancreatic tissue specimens [9]. Of particular relevance in the current context, Takayama *et al.* [10] demonstrated that APP is an androgen-regulated gene and that expression levels of the protein in PCa LNCaP cells positively correlated with cell proliferation. Higher APP protein expression level in PCa tissue specimens also correlated with a poorer disease prognosis. More recently, Miyazaki *et al.* [11] published data demonstrating that the proliferation and invasion of two PCa cell lines (LNCaP and DU145) were impaired following the depletion of the endogenous protein. The authors also demonstrated a down-regulation in EMT-related genes in these cell lines following APP-depletion.

In the current study, we have examined whether the over-expression of APP isoforms can promote EMT in DU145 cells and investigated the molecular prerequisites for such an effect. Our results show that APP₆₉₅ enhances EMT changes in an isoform-specific manner via a mechanism requiring intact E1 copper binding and cytosolic domains of the protein.

2. MATERIAL AND METHODS

2.1 Materials

Anti-APP C-terminal and anti-actin monoclonal antibodies were from Sigma-Aldrich (Poole, U.K.). Anti-APP 6E10 monoclonal antibody was from Cambridge Bioscience Ltd. (Cambridge, U.K.) and anti-sAPP β (1A9) antibody was kindly provided by Ishrut Hussain (GlaxoSmithKline, Harlow, U.K.). Monoclonal antibody 22C11 was from Millipore (Watford, U.K.). Anti-E-cadherin and anti-vimentin polyclonal antibodies were from R&D Systems (Minneapolis, U.S.A.). APP mutant DNA constructs were either synthesized in-house by site-directed mutagenesis of a wild-type APP₆₉₅ template or *de novo* by Epoch Life Science Inc. (Missouri City, U.S.A.). All other materials, unless otherwise stated, were from Sigma-Aldrich (Poole, U.K.).

85 2.2 Cell culture

86 All cell culture reagents were purchased from Lonza Ltd. (Basel, Switzerland). DU145 cells
87 were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with
88 25 mM glucose, 4 mM L-glutamine, 10% (v/v) foetal bovine serum, penicillin (50 U/ml),
89 streptomycin (50 mg ml⁻¹), and fungizone (2.5 mg ml⁻¹). Cells were maintained at 37°C in 5%
90 CO₂ in air. Stable DU145 transfectants were generated using the Amaxa cell line
91 Nucleofector Kit L in a Nucleofector 2b device (Lonza Ltd., Basel, Switzerland) and
92 subsequent selection of stable transfectants was performed using hygromycin B (200 µg ml⁻¹).
93 For the experiments involving the truncated sAPP α and sAPP β constructs, plasmids were
94 stably expressed in SH-SY5Y (sAPP α) or HEK (sAPP β) cells. Medium conditioned for 48 h
95 on mock-, sAPP α - and sAPP β -transfected cells was then harvested, centrifuged at 100, 000
96 g for 1 h, and concentrated 50-fold in centrifugal concentrators (Sartorius, Epsom, U.K.)
97 before being reconstituted to the original volume in Opti-MEM (Lonza Ltd., Basel,
98 Switzerland). This reconstituted, pre-conditioned medium was then incubated for 24 h with
99 mock-transfected DU145 cells before replacing with fresh pre-conditioned medium and
100 incubating for a further 24 h.

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102 2.3 Reverse transcription polymerase chain reaction (RT-PCR)

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104 Total RNA was extracted using an RNA extraction kit according to the manufacturer's
105 instructions (Qiagen, Crawley, U.K.) and RNA concentrations were determined using a
106 Nanodrop 2000c spectrophotometer reading at a wavelength of 260 nm (Thermo Scientific,
107 St Leon-Rot, Germany). Total RNA levels were equalized between samples and RT-PCR
108 reactions for APP isoforms and actin were performed using a Titanium One-step RT-PCR kit
109 (BD Biosciences, Oxford, U.K.) according to the manufacturer's instructions. Primers and
110 RT-PCR reaction conditions have been described previously [12].

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112 2.4 Protein assay

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114 Protein was quantified using bicinchoninic acid [13] in a microtitre plate with bovine serum
115 albumin (BSA) as a standard.

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117 2.5 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) 118 and immunoelectrophoretic blot analysis

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120 Samples were mixed with a half volume of reducing electrophoresis sample buffer and
121 boiled for 3 min. Proteins were resolved by SDS-PAGE using 7-17% polyacrylamide
122 gradient gels and transferred to Immobilon P polyvinylidene difluoride (PVDF) membranes
123 as previously described [14]. Anti-APP C-terminal antibody detects an epitope within amino
124 acids 676-695 (APP₆₉₅ numbering) of the APP cytosolic domain and, therefore, detects all
125 full-length APP isoforms; it was used at a dilution of 1:7500. Anti-APP 6E10 detects an
126 epitope within amino acid residues 3-8 of the A β region of APP and, therefore, detects both
127 full-length and sAPP α forms of all three major APP isoforms; it was used at a dilution of
128 1:2500. Anti-sAPP β (1A9) detects a neoepitope formed at the C-terminus of sAPP β following
129 β -secretase cleavage of full-length APP and, therefore, specifically detects the sAPP β forms
130 of all three major APP isoforms; it was used at 1:3000. Antibody 22C11 detects an epitope
131 within amino acid residues 66-81 of the N-terminal ectodomain of APP and, therefore,
132 detects all soluble and full-length forms of all three major APP isoforms; it was used at
133 1:1000. Anti-actin was used at 1:5000 whilst the anti-E-cadherin and anti-vimentin antibodies
134 were both used at 1:2000. Bound antibody was detected using peroxidase-conjugated
135 secondary antibodies (Sigma-Aldrich, Poole, U.K. and R&D Systems Europe Ltd., Abingdon,
136 U.K.) in conjunction with enhanced chemiluminescence detection reagents (Perbio Science
137 Ltd, Cramlington, U.K.).

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139 2.6 Light microscopy and dendricity analysis

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141 A Nikon (Surrey, U.K.) eclipse TE200 optical microscope was used in conjunction with a
142 Nikon COOLPIX P6000 digital camera to produce cell images. Dendricity factor analysis was
143 performed by calculating the area and perimeter of ten randomly selected cells (using points

of a superimposed grid to choose individual cells for analysis) using image J software. These data points were then utilised to calculate dendricity factor using the equation $P^2/4\pi A$ when P = perimeter of cell and A = area of cell.

2.7 Statistical analysis

All data are presented as the means $\pm$ S.D. Data were subjected to statistical analysis via Student's t-test. Levels of significance are indicated in the figure legends.

3. RESULTS AND DISCUSSION

Initially we examined which APP isoforms were endogenously expressed in DU145 cells using semi-quantitative RT-PCR (measurements at the protein level in cell lysates are hindered by the existence of multiple co-migrating glycoforms of each isoform). Note that this experiment was not designed to accurately quantify the individual APP isoforms merely to determine which isoforms were present for subsequent over-expression studies. Therefore, it was not necessary to perform real time quantitative PCR. The results (Fig. 1A) revealed three main RT-PCR products generated from the DNA of mock (vector only)-transfected DU145 cells. These bands, at 562, 505 and 337 base pairs, corresponded to the expected product sizes for APP₇₇₀, APP₇₅₁ and APP₆₉₅, respectively. The identity of the smallest band was confirmed as APP₆₉₅ by conducting the same experiment using template DNA isolated from APP₆₉₅-transfected DU145 cells. Note that slight increases in the larger APP isoform products were also observed in APP₆₉₅-transfected cells; we believe that this is due to the weak ability of transfected APP₆₉₅ to self-regulate the expression of the endogenous protein as observed previously [15]. Although only semi-quantitative, these data were sufficient to demonstrate that the main APP isoforms present in DU145 cells are APP₇₇₀ and APP₇₅₁, with lesser amounts of APP₆₉₅.

Given the lower endogenous background levels of APP₆₉₅, we initially examined the effects of over-expressing this isoform on EMT. Following the stable transfection of DU145 cells, full-length over-expressed APP₆₉₅ was detected in cell lysates by immunoblotting as two main bands at around 110 kDa (representing the immature and fully glycosylated mature forms of the protein, respectively) (Fig. 1B). The α -secretase-cleaved forms of APP₇₅₁ and APP₇₇₀ (sAPP α) in conditioned medium co-migrated as a single band whilst transfected APP₆₉₅ was detected as a slightly smaller band migrating below the larger protein isoforms (Fig. 1C). Note that only mature APP reaches the cell surface and is subsequently cleaved by secretase activity such that the detection of the different isoforms in conditioned medium is not confused by the presence of multiple glycoforms. Although roughly equivalent amounts of sAPP α were generated from APP₆₉₅ and the larger protein isoforms, we observed, as others have done so previously [16], that sAPP β was generated almost exclusively from the smaller isoform (Fig. 1C).

Having confirmed the over-expression of APP₆₉₅, we next examined the effects of the protein on EMT in DU145 cells. Light microscopy images revealed EMT-like changes in morphology following APP₆₉₅ transfection with cells exhibiting a more independent growth pattern with many more membrane protrusions (Fig. 2A). As would be expected following EMT [4], levels of E-cadherin decreased 60% in APP₆₉₅-transfected cells whilst vimentin levels increased 4.6-fold (Fig. 2B).

We next examined the APP molecular prerequisites for the promotion of EMT by generating a range of APP constructs (Fig. 3). Immunoblot data documenting the relative expression and generation of proteolytic fragments from these constructs have been reported previously by our group [17] and, therefore, are presented only in summarised table format in the current manuscript (Table 1). Initially we examined whether the larger APP₇₅₁ and APP₇₇₀ isoforms could elicit EMT in a similar manner to their smaller APP₆₉₅ counterpart (Fig. 3A). These holoproteins were over-expressed in DU145 cells to the same level as APP₆₉₅ and the levels of sAPP α generated were not significantly different (Table 1). In contrast, the levels of sAPP β generated from the two larger isoforms were significantly less than from APP₆₉₅ (Table 1). Changes in EMT were quantified in terms of the relative dendricity of the different cell lines (see Materials and Methods section). APP₆₉₅ enhanced dendricity by 153% relative

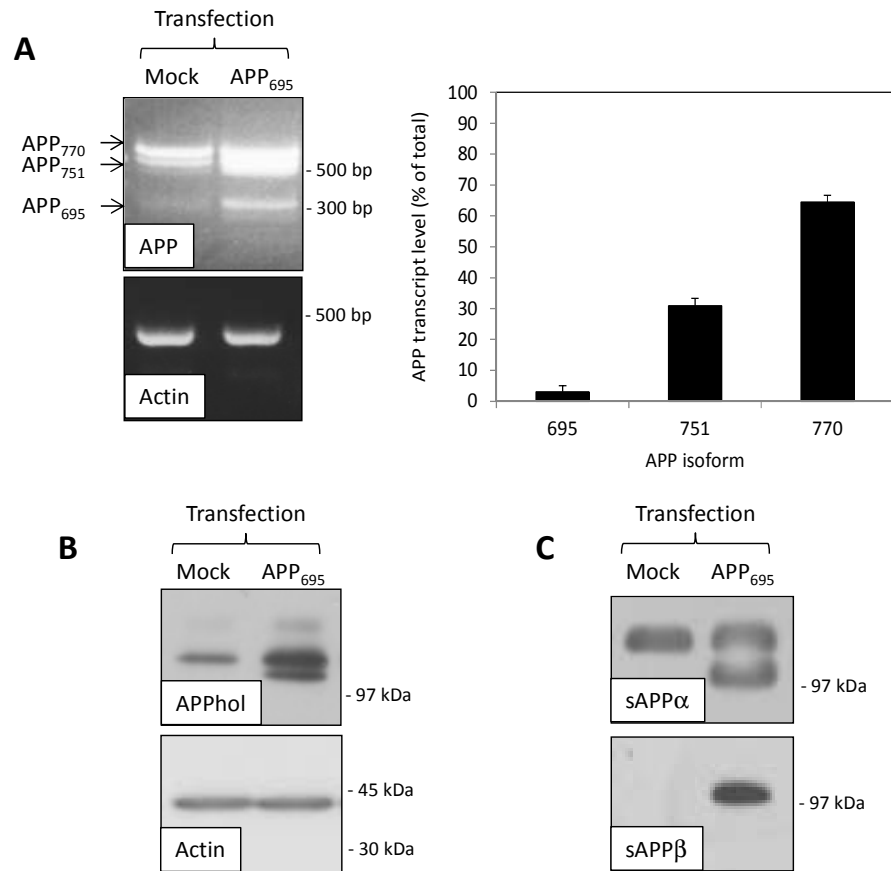
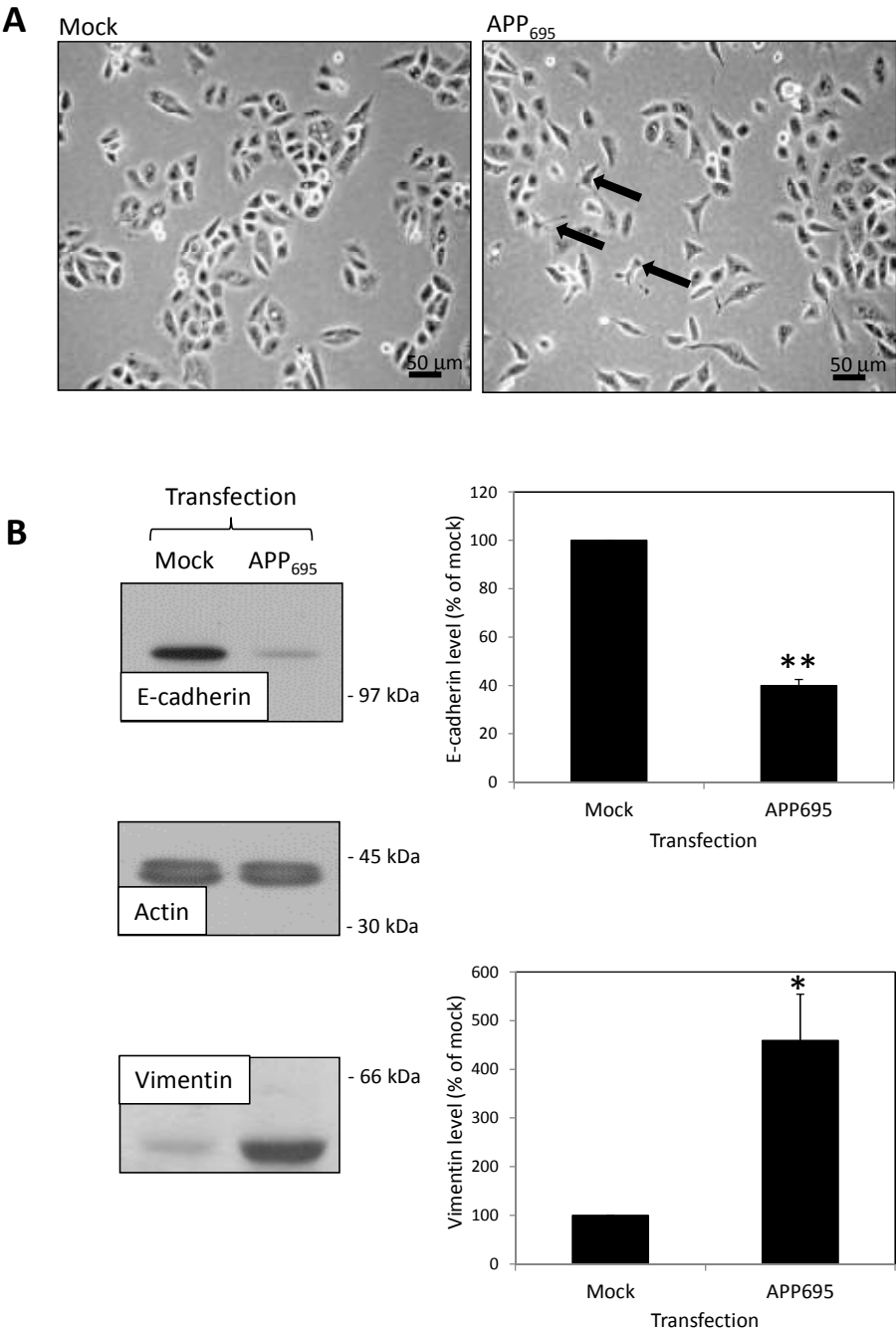


Figure 1. Expression and proteolysis of APP in DU145 cells.

A. Semi-quantitative RT-PCR analysis of APP isoform transcript levels in mock- (vector only) and APP₆₉₅-transfected DU145 cells. A graphical quantification of the relative levels of endogenous DU145 APP transcripts is shown; results are means $\pm$ S.D. (n=3). An actin RT-PCR is also shown in order to confirm equal template DNA concentrations in the reactions. **B.** Immunoblot analysis of APP holoprotein and actin levels in mock- and APP₆₉₅-transfected DU145 cell lysates. **C.** Immunoblot analysis of sAPP α and sAPP β levels in conditioned medium from mock- and APP₆₉₅-transfected DU145 cells.

to mock-transfected cells (Table 1) whereas the two larger APP isoforms had no significant effect. Thus, the ability to promote EMT was specific to the APP₆₉₅ isoform and, as such, all further constructs investigated were based on this smaller isoform.

As the amount of sAPP β generated from APP₆₉₅ was greater than from APP₇₅₁ or APP₇₇₀, we postulated that the soluble forms of the protein generated by α - or β -secretase activity might be capable of promoting EMT. Therefore, we generated constructs analogous to these fragments truncated C-terminally to lysine612 (sAPP α) and methionine596 (sAPP β) (Fig. 3B and 3C). Unfortunately, when the former construct was expressed in DU145 cells, the product was aberrantly processed intracellularly (Fig. 4A and 4B). However, when expressed in alternative cell lines (HEK or SH-SY5Y) the two constructs were secreted as the predicted fragments of approximately 110 kDa (Fig. 4C and 4D). We, therefore, incubated mock-transfected DU145 cells with conditioned medium from these alternate cell lines (see Materials and Methods section) but both constructs failed to promote EMT (Table 1). Also of note is the fact that α - and β -secretase inhibitors had no effect on the morphology of APP₆₉₅-transfected DU145 cells (data not shown).



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Figure 2. APP₆₉₅ promotes epithelial to mesenchymal transition in DU145 cells.
A. Microscopy images of mock- and APP₆₉₅-transfected DU145 cells. The arrows on the right hand image are incorporated in order to highlight some of the more dramatic morphological changes observed in the APP₆₉₅-transfected cells. **B.** Immunoblot analysis of E-cadherin and vimentin levels in mock- and APP₆₉₅-transfected DU145 cell lysates. Graphical quantification of the relative levels of the two proteins are also shown; results are means $\pm$ S.D. (n=3). * and ** denote significance at $P = .01$ and $P = .005$, respectively. An actin immunoblot is also shown in order to confirm equal total protein levels between samples.

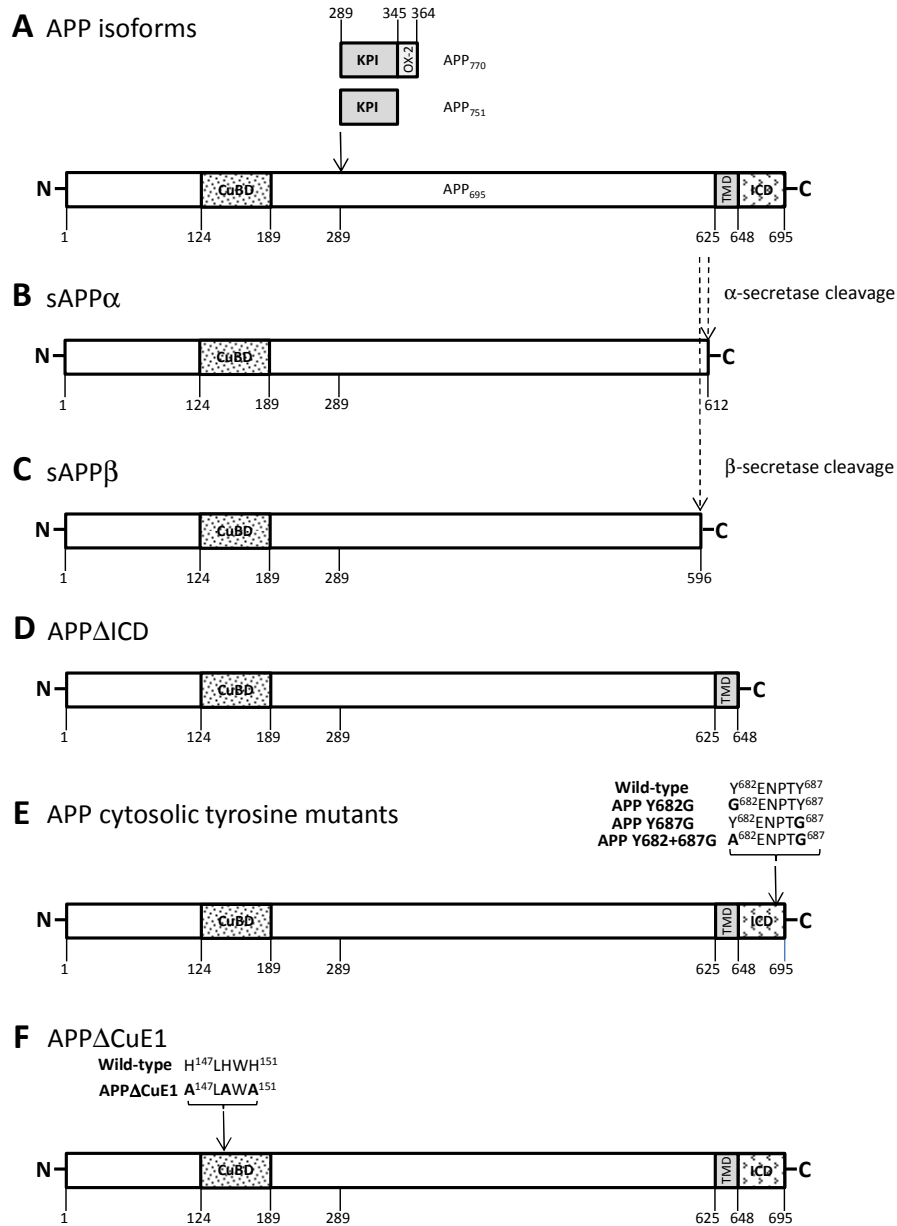


Figure 3. A schematic detailing the APP constructs employed.

A. The APP₇₇₀ isoform possesses both Kunitz protease inhibitor (KPI) and OX-2 domains between residues 289 and 364. APP₇₅₁ lacks the latter domain and APP₆₉₅ lacks both domains. All additional constructs were based on the APP₆₉₅ isoform. **B.** The sAPP α construct is analogous to soluble APP cleaved from the holoprotein by α -secretase activity and is, therefore, truncated C-terminally to lysine 612. **C.** The sAPP β construct is analogous to soluble APP cleaved from the holoprotein by β -secretase activity and is, therefore, truncated C-terminally to methionine 596. **D.** The APP Δ ICD construct is truncated after the transmembrane domain (TMD) and, therefore, lacks the intracellular domain (ICD) of the wild-type protein. **E.** The APP cytosolic tyrosine mutants have tyrosine to glycine mutations in the ICD at positions 682 (APP Y682G), 687 (APP Y687G) or at both of these residues (APP Y682G+687G). **F.** The APP Δ CuE1 construct possesses three histidine to alanine mutations at positions 147, 149 and 151 within the E1 copper binding domain (CuBD) of the protein.

Table 1. Expression and proteolysis of APP constructs and their effects on the relative dendricity of DU145 cells.

*The expression and proteolytic fragment formation levels are expressed relative to those detected in APP₆₉₅-transfected cells. The relative dendricity results are expressed relative to mock (vector only)-transfected cells. Results are means ± S.D. (n=3). N/A = not applicable. Statistically significant differences are in bold type. * and ** denote significance at P = .01 and P = .005, respectively.*

Construct transfected	Uncleaved protein in cell lysates	sAPPα in conditioned medium	sAPPβ in conditioned medium	Relative dendricity
Mock (empty vector)	N/A	N/A	N/A	100%
APP ₆₉₅	100%	100%	100%	153.0 ± 16.6 %*
APP ₇₅₁	98.2 ± 4.6 %	97.6 ± 8.7 %	36.7 ± 12.1 %*	99.9 ± 5.6 %
APP ₇₇₀	102.1 ± 10.2 %	98.3 ± 13.2 %	53.6 ± 13.9 %*	102.8 ± 3.7 %
sAPPα	N/A	362.5 ± 38.9 %**	N/A	86.3 ± 14.4 %
sAPPβ	N/A	N/A	465.3 ± 67.3 %**	98.1 ± 5.9 %
APPΔICD	87.3 ± 19.4 %	93.9 ± 21.4 %	0	105.4 ± 10.1 %
APP Y682G	97.4 ± 18.3 %	101.8 ± 12.5 %	36.7 ± 21.2 %*	161.2 ± 33.1 %*
APP Y687G	101.3 ± 21.6 %	89.2 ± 19.9 %	56.1 ± 12.3 %**	97.1 ± 8.2 %
APP Y682+687G	89.9 ± 20.5 %	95.0 ± 16.7 %	24.1 ± 11.3 %**	96.2 ± 7.8 %
APPΔCuE1	112.6 ± 17.3 %	97.0 ± 12.9 %	101.2 ± 21.6 %	81.5 ± 18.5 %

The fact that soluble forms of APP failed to promote EMT suggested that the intracellular domain (ICD) of the protein might be a prerequisite in this respect. Therefore, we generated a construct (APPΔICD) truncated C-terminally to residue 648 (Fig. 3D). Following transfection into DU145 cells, the expression level of APPΔICD was identical to that of wild-type APP₆₉₅ as was the level of sAPPα generated from the two proteins (Table 1). However, no sAPPβ was generated from the former construct which was probably indicative of the fact that the APP ICD is required for internalisation of the protein and subsequent β-secretase cleavage. However, given our previous results showing that sAPPβ had no effect on EMT, the fact that the APPΔICD construct failed to promote the process (Table 1) was almost certainly due to the lack of intracellular domain rather than a change in sAPPβ generation.

In order to narrow down the involvement of the APP ICD in EMT, we examined the role of two cytosolic tyrosine residues within this domain (tyrosines682 and 687) the phosphorylation of which might be linked to cell signalling. Three constructs were generated; APP Y682G, APP Y687G and APP Y682+687G (Fig. 3E) and the corresponding holoproteins were found to express to similar levels as that of wild-type APP₆₉₅ in DU145 cells (Table 1). Whilst the levels of sAPPα generated from these constructs were not statistically different to that generated from the wild-type protein, the levels of sAPPβ were reduced (Table 1). Interestingly, the single Y682A mutant promoted EMT to a similar extent as wild-type APP₆₉₅ (Table 1), despite a decreased generation of sAPPβ, adding further support to the fact that the generation of this fragment was not a prerequisite. However, both

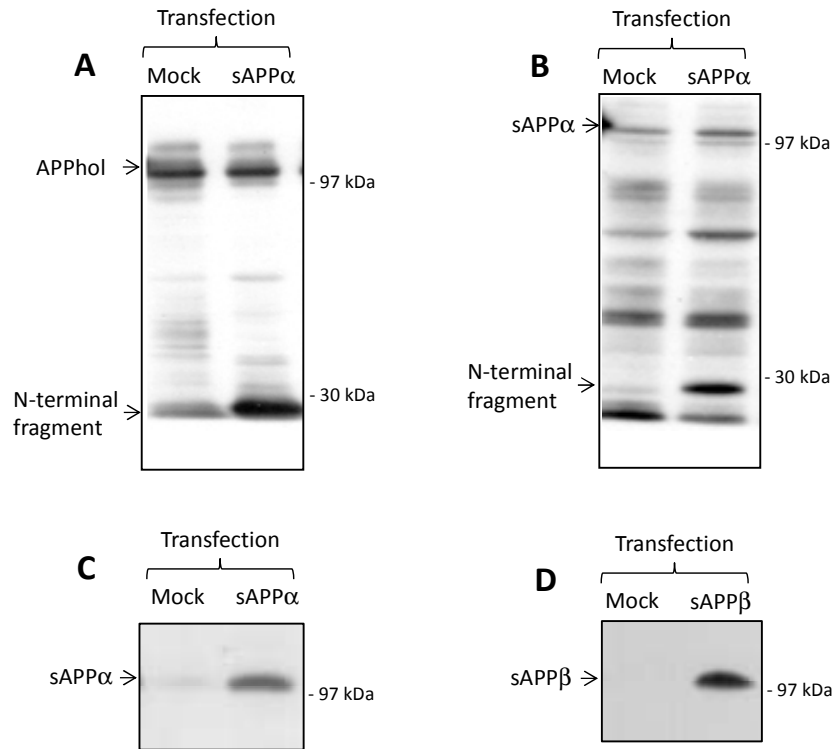


Figure 4. Soluble APP construct expression.

Stable expression of the sAPP α construct in DU145 cell lysates (A) and conditioned medium (B). APP and its proteolytic fragments were detected using antibody 22C11. The over-expressed construct was detected as an unexpected N-terminal fragment at 25 kDa in both lysates and conditioned medium.

Levels of sAPP α at the expected molecular mass of 110 kDa were not changed significantly. C. Detection of the sAPP α construct in pre-conditioned medium from stably transfected SH-SY5Y cells using anti-APP antibody 6E10. D. Detection of the sAPP β construct in pre-conditioned medium from stably transfected HEK cells using antibody 1A9. Note that, in the two latter panels, the over-expressed sAPP was detected at the correct predicted molecular mass for the fragments.

the APP Y687A and the APP Y682+687A mutants were unable to promote EMT indicating that the tyrosine residue at position 687 but not at position 682 was required in this respect.

Finally, we examined the role of the copper binding domain (CuBD) in the E1 extracellular domain of APP in relation to EMT. We generated a construct (APP Δ CuE1) in which three histidines were mutated to alanine in a key area of the protein associated with copper binding [18] (Fig. 3F). This construct was expressed and processed in DU145 cells in a manner identical to that of wild-type APP₆₉₅ (Table 1). However, APP Δ CuE1 failed to promote EMT indicating that the extracellular E1 CuBD, in addition to the cytosolic domain, of APP were prerequisites in this respect.

4. CONCLUSION

Taken together, our findings show for the first time that the 695 amino acid isoform of APP is capable of promoting EMT in a prostate cancer cell line. The mechanism of action is likely to involve signalling via the cytosolic domain of the protein and also the E1 copper binding region within the extracellular domain of the protein. However, it is clear that, whilst these

factors are prerequisites for APP-induced EMT, there must be additional properties specific to APP₆₉₅ that participate in the phenomenon given the fact that the larger APP₇₅₁ and APP₇₇₀ isoforms do not act in a similar manner. Thus, the repression of APP₆₉₅ expression may represent a therapeutic strategy for reducing the metastatic potential of prostate cancer cells.

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COMPETING INTERESTS

The authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

Author EP designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. All authors managed the analyses of the study and read and approved the final manuscript.

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459 ABBREVIATIONS

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461 A β , amyloid beta; AD, Alzheimer's disease; APP, amyloid precursor protein; BACE1, beta-
462 site APP-cleaving enzyme 1; CuBD, copper binding domain; EMT, epithelial to
463 mesenchymal transition; ICD, intracellular domain; PCa, prostate cancer; RT-PCR, reverse
464 transcription polymerase chain reaction; sAPP α , soluble APP alpha; sAPP β , soluble
465 APPbeta; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

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