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The E1 copper binding domain of full-length amyloid precursor protein mitigates copper-induced growth inhibition in brain metastatic prostate cancer DU145 cells

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ABSTRACT

Copper plays an important role in the aetiology and growth of tumours and levels of the metal are increased in the serum and tumour tissue of patients affected by a range of cancers including prostate cancer (PCa). The molecular mechanisms that enable cancer cells to proliferate in the presence of elevated copper levels are, therefore, of key importance in our understanding of tumour growth progression. In the current study, we have examined the role played by the amyloid precursor protein (APP) in mitigating copper-induced growth inhibition of the PCa cell line, DU145. A range of APP molecular constructs were stably over-expressed in DU145 cells and their effects on cell proliferation in the presence of copper were monitored. Our results show that endogenous APP expression was induced by sub-toxic copper concentrations in DU145 cells and over-expression of the wild-type protein was able to mitigate copper-induced growth inhibition via a mechanism involving the cytosolic and E1 copper binding domains of the full-length protein. APP likely represents one of a range of copper binding proteins that PCa cells employ in order to ensure efficient proliferation despite elevated concentrations of the metal within the tumour microenvironment. Targeting the expression of such proteins may contribute to therapeutic strategies for the treatment of cancers.

1. Introduction

The role of copper in the aetiology and growth of tumours has been extensively studied over the last two decades based initially on reports that serum and tumour levels of the metal are significantly elevated in cancer patients compared to healthy subjects [1]. At the molecular level, copper may contribute to cancer progression via a number of mechanisms including oxidative stress-mediated cell damage or the stimulation of angiogenesis [1]. Thus, the mechanisms by which cancer cells might regulate their resistance to enhanced copper levels within the tumour microenvironment are of great importance to disease development.

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, APP_{695}, APP_{751} and APP_{770} [2]. The main components of the extracellular senile plaques in the AD-afflicted brain are amyloid beta (Aβ)-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 [2]. The initial soluble product generated by β-secretase is termed sAPPβ (soluble APP beta). In contrast to this 'amyloidogenic' pathway, APP is also processed via a non-amyloidogenic route involving α-secretase cleavage within the Aβ domain [2]. This latter cleavage precludes Aβ-peptide formation and generates a sAPPα (soluble APP alpha) fragment.

More recently, APP has been shown to play a role in cancer. Expression of the protein stimulates colon carcinoma cell proliferation [3] and an increase in APP mRNA expression in oral squamous cell carcinomas is associated with a reduction in patient survival [4]. Thyroid cancers are characterised by up regulation of APP protein and mRNA expression [5] with the former also being enhanced in pancreatic cancer tissue specimens [6].

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; APP, amyloid precursor protein; BACE1, beta-site APP-cleaving enzyme 1; CuBD, copper binding domain; ICD, intracellular domain; PCa, prostate cancer; sAPPα, soluble APP alpha; sAPPβ, soluble APP beta; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

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Of particular relevance to the current study, APP has previously been shown to enhance the proliferation of the prostate cancer (PCa) cell line, LNCaP [7]. More recently, Miyazaki et al. [8] published data in this journal demonstrating that the proliferation and invasion of two PCa cell lines (LNCaP and DU145) were impaired following depletion of the endogenous protein.

APP is a copper binding protein that has been implicated in cellular copper homeostasis [9] and several reports exist specifically demonstrating increased copper levels in the whole blood, plasma or serum of prostate cancer (PCa) patients [10–12]. Therefore, in the current study, we have investigated the ability of APP to mitigate the copper-induced growth inhibition of the brain metastatic PCa cell line, DU145. Although metastasis of PCa cells to the brain is a relatively uncommon event, it is increasing in prevalence probably as a consequence of increased patient survival times due to new drug regimes. Furthermore, the brain exhibits high copper concentrations second only to the liver [13] making DU145 cells a particularly appropriate cell line for the current type of study. Our results show that the endogenous expression of APP was enhanced by copper in DU145 cells and that the over-expression of any of the three major APP isoforms mitigated copper-induced growth inhibition. Furthermore, we have shown that membrane anchorage of APP and intact cytosolic and E1 copper binding domains are all molecular pre-requisites in this respect. Thus, APP might represent one of a range of copper binding proteins utilised by cancer cells to promote growth in the presence of elevated copper concentrations within the tumour microenvironment.

2. Materials 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-sAPPβ (1A9) antibody was kindly provided by Ishrut Hussain (GlaxoSmithKline, Harlow, U.K.). APP mutant DNA constructs were either synthesized in-house by site-directed mutagenesis of a wild-type APP_{695} 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.).

2.2. Cell culture

All cell culture reagents were purchased from Lonza Ltd. (Basel, Switzerland). DU145 cells were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 25 mM glucose, 4 mM L-glutamine, 10% (v/v) foetal bovine serum, penicillin (50 U/ml), streptomycin (50 mg ml^{-1}), and fungizone (2.5 mg ml^{-1}). Cells were maintained at 37 °C in 5% CO_2 in air. Stable DU145 transfectants were generated using the Amaxa cell line Nucleofector Kit L in a Nucleofector 2b device (Lonza Ltd., Basel, Switzerland) and subsequent selection of stable transfectants was performed using hygromycin B (200 μg ml^{-1}). For the experiments involving the truncated sAPPα and sAPPβ constructs, plasmids were stably expressed in SH-SY5Y (sAPPα) or HEK (sAPPβ) cells. Medium conditioned for 48 h on mock-, sAPPα- and sAPPβ-transfected cells was then harvested, centrifuged at 100,000*g* for 1 h, and concentrated 50-fold in centrifugal concentrators (Sartorius, Epsom, U.K.) before being reconstituted to the original volume in Opti-MEM (Lonza Ltd., Basel, Switzerland). This reconstituted, pre-conditioned medium was then used to culture DU145 cells in the absence or presence of copper for 7 days with the medium being replaced with fresh pre-conditioned medium every two days. Copper (either free $CuCl_2$ or glycine:copper complexes) was pre-incubated at the indicated final concentrations for 1 h with complete growth medium prior to the addition of the medium to cells.

2.3. Cell viability assay

Cell proliferation was examined using methanethiosulfonate (MTS) (Promega, Wisconsin, U.S.A.) or by counting cells using Trypan blue staining. At the time points indicated, cells were incubated with CellTiter 96® AQueous One Cell Proliferation Assay (MTS) solution for 2 h at 37 °C. Absorbance readings at 492 nm were then taken using an Anthos 2020 microplate reader (Perkin-Elmer, Massachusetts, U.S.A.).

2.4. Protein assay, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblot analysis

Protein was quantified using bicinchoninic acid [14] in a microtitre plate with bovine serum albumin as a standard. Proteins were resolved by SDS–PAGE and transferred to Immobilon P polyvinylidene difluoride membranes as previously described [15]. Anti-APP C-terminal antibody was used at a dilution of 1:7500, anti-actin at 1:5000, anti-APP 6E10 at 1:2500 and anti-sAPPβ (1A9) at 1:3000. Bound antibody was detected using peroxidase-conjugated secondary antibodies (Sigma–Aldrich, Poole, U.K. and R&D Systems Europe Ltd., Abingdon, U.K.) in conjunction with enhanced chemiluminescence detection reagents (Perbio Science Ltd, Cramlington, U.K.).

2.5. Statistical analysis

All data are presented as the means ± S.D. or S.E.M. Data were subjected to statistical analysis via Student's *t*-test. Levels of significance are indicated in the figure legends.

3. Results and discussion

3.1. Copper inhibits DU145 cell proliferation and enhances endogenous APP expression

Initially we examined the inhibitory effect of copper on the growth of untransfected DU145 cells by culturing them in complete growth medium containing various metal concentrations (results were identical whether metal was added to growth medium in the form of free $CuCl_2$ or glycine-complexed copper). DU145 cells were fundamentally very resistant to copper with no significant inhibition of growth being apparent at metal concentrations of 100 μM and lower (Fig. 1A). At 150 μM copper, growth inhibition became apparent at 4 days and persisted for the entire 7 day growth period whilst very little growth occurred at all in the presence of 200 μM copper. The authors concede that levels of copper within the tumour microenvironment are unlikely to exceed 30 μM [1] at which concentration tumour cells are clearly able to maintain copper homeostasis through a range of possible mechanisms [16]. However, it is only when these mechanisms are overloaded at higher metal concentrations (as in the current study) that individual elements of these mechanisms can be tested.

We next examined whether the expression of endogenous APP could be enhanced by copper as observed previously in non-cancerous cell lines [17]. Immunoblot analysis of lysates prepared from DU145 cells cultured in the presence of various copper concentrations demonstrated that APP expression was enhanced at metal concentrations as low as 10 μM (Fig. 1B) and peaked at 50 μM; at which point levels of the protein were increased 2.74 ± 0.48-fold relative to those in lysates from untreated cells (Fig. 1C). Thus, it is possible that DU145 cells increase their

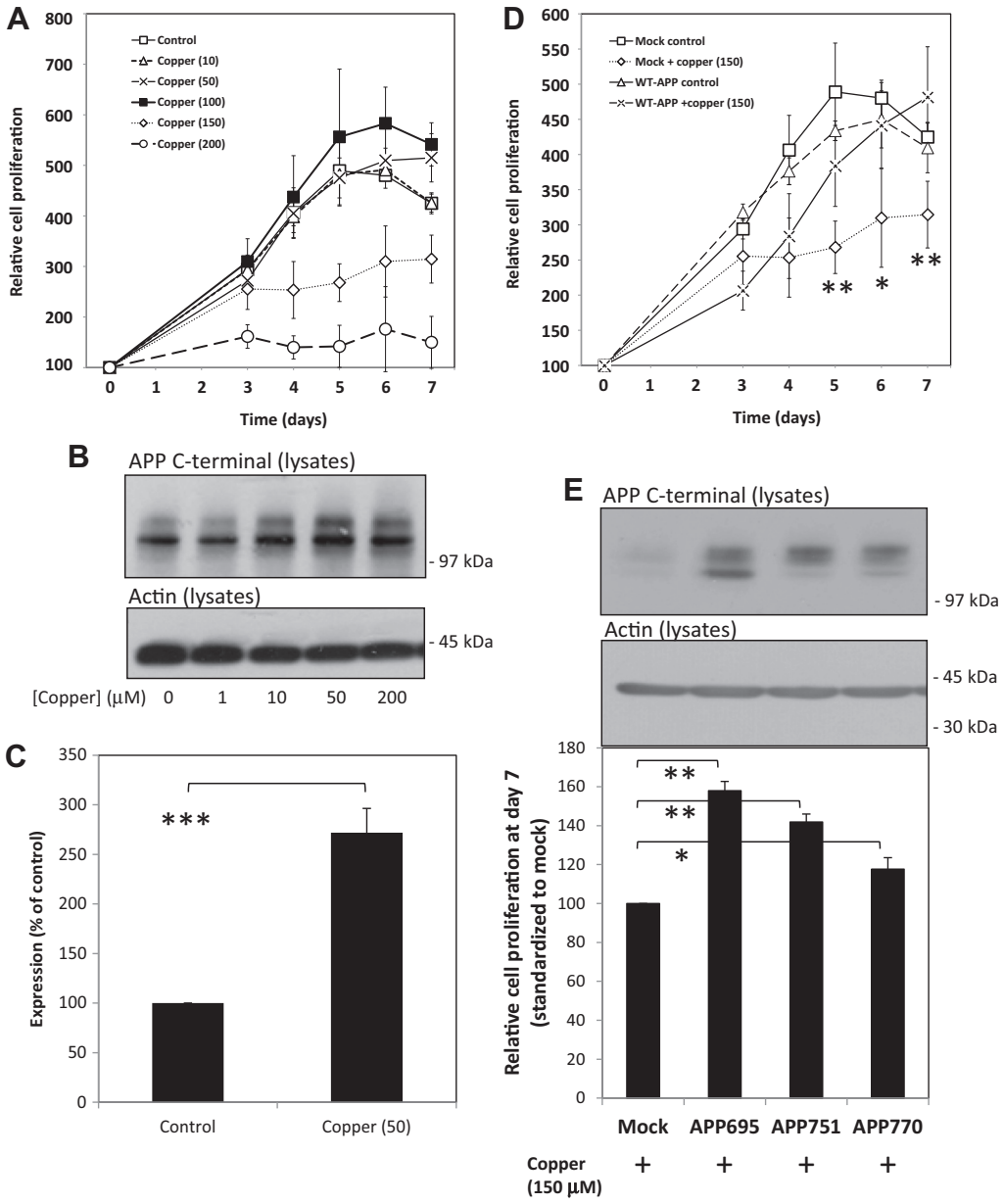

Fig. 1. Copper-induced growth inhibition and wild-type APP expression in DU145 cells. (A) Untransfected DU145 cells were cultured over a 7 day period in the absence/presence of the indicated copper concentrations. At various time points, cell viability was assayed as described in the Section 2. Results are expressed relative to viability at day 0. (B) Immunoblot of APP and actin expression in lysates prepared from untransfected DU145 cells cultured for 7 days in medium containing the indicated copper concentrations. (C) Quantification of endogenous APP expression in DU145 cells cultured for 7 days in the absence or presence of copper (50 μM). Results are expressed relative to APP expression levels in the untreated control (no metal). (D) DU145 cells stably transfected with either vector alone (Mock) or wt-APP_{695} (WT-APP) were cultured over a 7 day period in the absence or presence of copper (150 μM). At the various time points, cell viability was assayed as described in the Section 2. Results are expressed relative to viability at day 0. (E) DU145 cells stably transfected with either vector alone (Mock) or the indicated APP isoform were cultured over a 7 day period in the presence of copper (150 μM). At 7 days lysates were prepared and immunoblotted for APP and actin and cell viability was determined as described in the Section 2. Results are expressed relative to the viability of Mock-transfected cells cultured in the presence of copper at day 7. All results are means ± S.D. ($n = 3$). *, ** and *** Denote significance at $P = 0.05$, $P = 0.01$ and $P = 0.005$, respectively.

expression of APP as part of a defence against copper-induced cytotoxicity.

3.2. Wild-type APP mitigates copper-induced growth inhibition in DU145 cells regardless of isoform type

Having determined the threshold concentration at which copper inhibited DU145 cell growth, we next sought to examine whether the over-expression of wild-type (wt)-APP could mitigate this effect. DU145 cells stably transfected with either vector alone (Mock) or wt-APP_{695} were cultured in the absence or presence of 150 μM copper. The results (Fig. 1D) show that APP_{695} did not promote growth in the absence of exogenous copper. However, whilst the growth of mock-transfected cells was inhibited by copper, the over-expression of APP_{695} completely mitigated this effect. As DU145 cells mainly express the larger APP_{751} and APP_{770} isoforms (with lower levels of APP_{695}), we also examined the isoform specificity of this phenomenon. Following the generation of stable transfectants, all three APP isoforms were found to be over-expressed at similar levels (Fig. 1E). Furthermore, all three isoforms increased cell growth in the presence of copper (150 μM) (Fig. 1E).

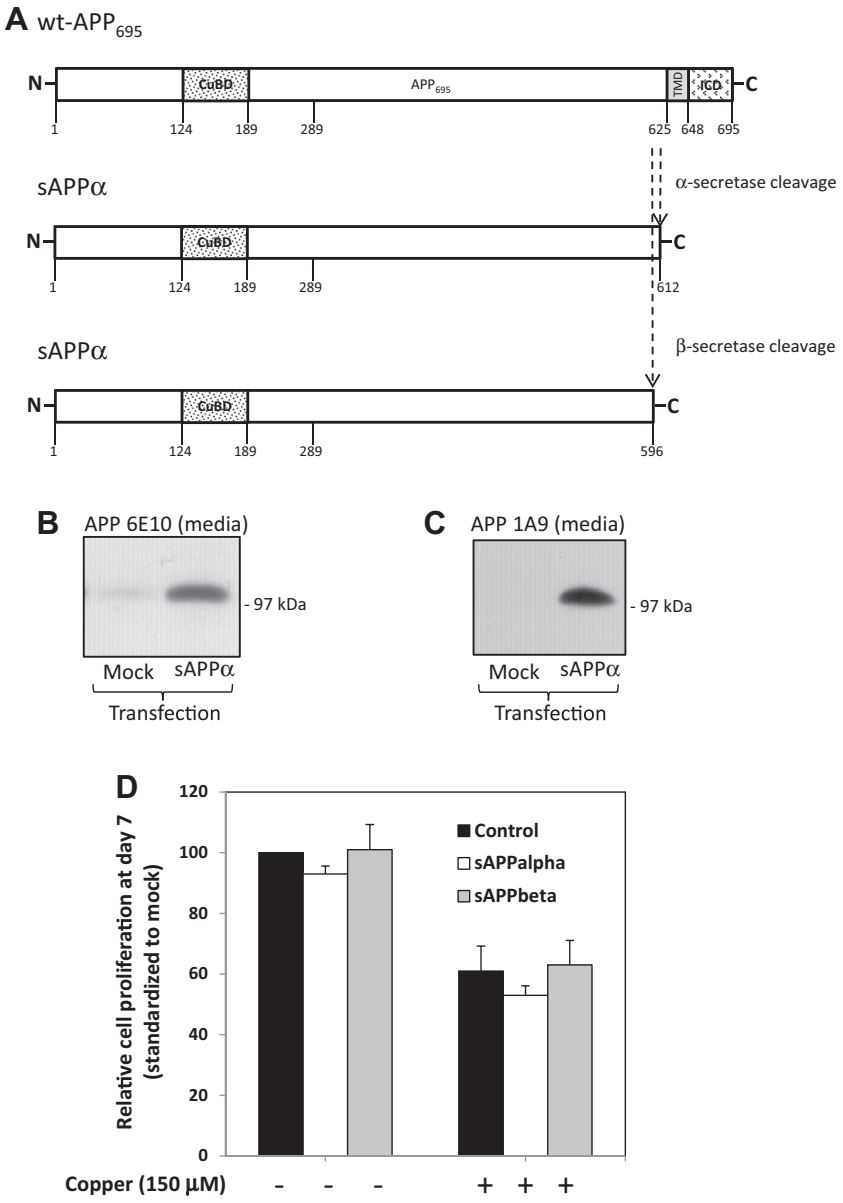

Fig. 2. The effect of soluble APP constructs on the growth of DU145 cells in the absence and presence of exogenous copper. (A) Schematic detailing the APP constructs employed. All constructs were based on the 695 amino acid isoform of wild-type (wt)-APP. The sAPPα construct is analogous to soluble APP cleaved from the holoprotein by α-secretase activity and is, therefore, truncated C-terminally to lysine 612. The sAPPβ construct is analogous to soluble APP cleaved from the holoprotein by β-secretase activity and is, therefore, truncated C-terminally to methionine 596. CuBD, copper binding domain; ICD, intracellular domain; TM, transmembrane domain. (B) Immunoblot of sAPPα levels in conditioned medium prepared from Mock- and sAPPα-transfected SH-SY5Y cells. (C) Immunoblot of sAPPβ levels in conditioned medium prepared from Mock- and sAPPβ-transfected HEK cells. (D) Untransfected DU145 cells were cultured in pre-conditioned media (see Section 2) from Mock-, sAPPα- or sAPPβ-transfected SH-SY5Y or HEK cells in the presence or absence of copper (150 µM). At 7 days, cell viability was determined as described in the Section 2. Results are expressed relative to the viability of DU145 cells cultured in the presence of medium pre-conditioned on Mock-transfected cells (no exogenous metal) and are means ± S.D. (n = 3).

It is interesting to note that, in the current over-expression study, we did not find that APP increased DU145 cell proliferation in the absence of exogenous copper. *Prima facie*, this finding might seem at odds with recent data reported by Miyazaki et al. [8] which showed that the depletion of endogenous APP using siRNA in DU145 cells reduced cell proliferation. One possible explanation for this apparent discrepancy might be that only a threshold level of endogenous APP expression is required to promote cell growth in the absence of copper such that enhancing expression further by APP transfection has no additive effect on growth. Only when the cells are exposed to copper insult might additional APP be required to mitigate the resultant metal-induced growth inhibition.

3.3. Soluble forms of APP are not sufficient to mitigate copper-induced growth inhibition

Next we sought to determine which parts of the APP molecule were prerequisites for the mitigation of copper-induced growth inhibition. Initially we examined whether soluble forms of the protein could exert a similar effect to that of full-length APP using constructs analogous to sAPPα (truncated C-terminally to lysine612) and sAPPβ (truncated C-terminally to methionine 596) (Fig. 2A). Note that all molecular constructs were based on the 695 amino acid APP isoform. Unfortunately, when these soluble constructs were expressed in DU145 cells, the products were aberrantly processed intracellularly (data not shown). However, when

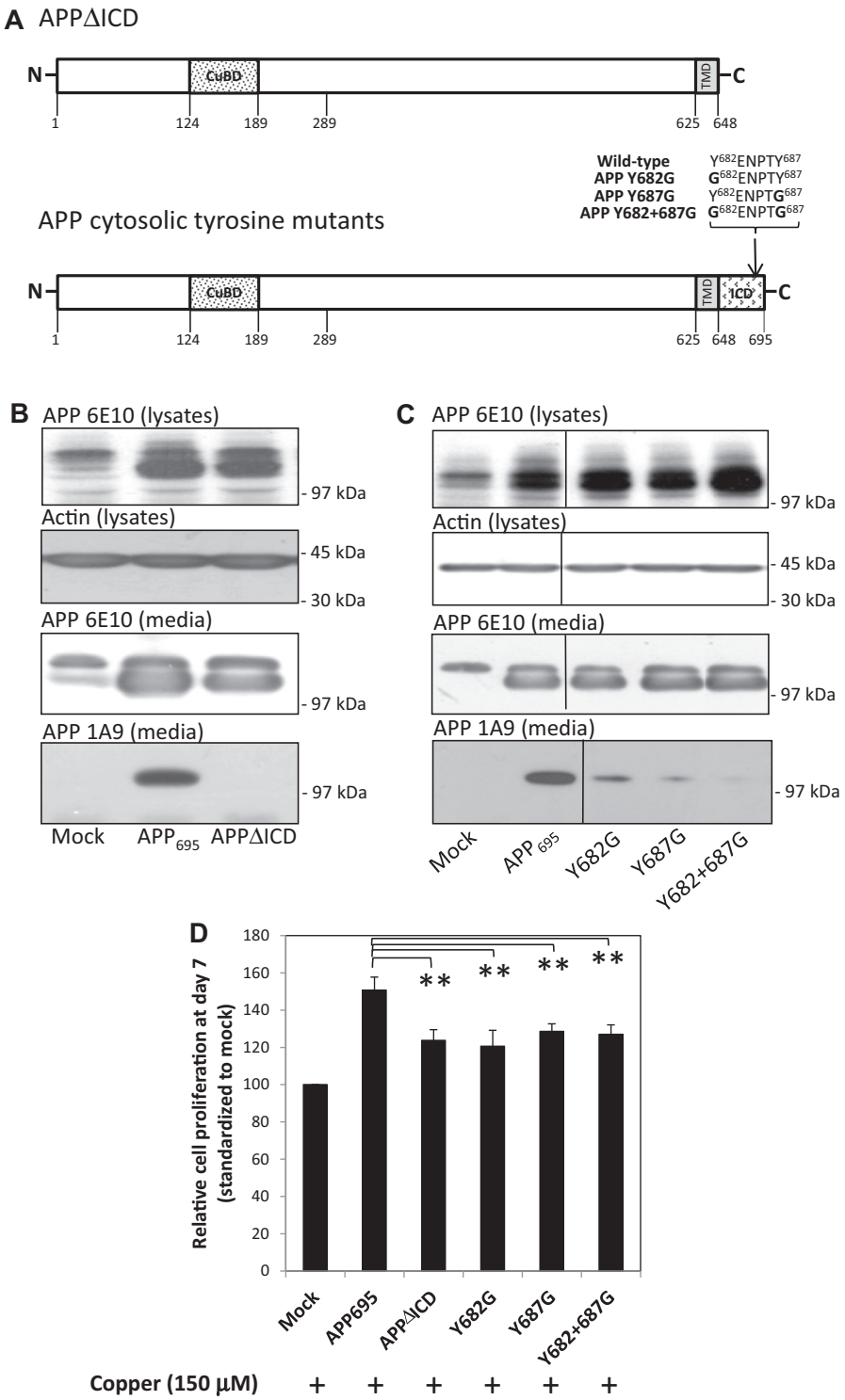

Fig. 3. The effect of intracellular domain APP constructs on the growth of DU145 cells in the presence of copper. (A) Schematic detailing the APP constructs employed. The APPΔICD construct is truncated after the transmembrane domain (TMD) and, therefore, lacks the intracellular domain (ICD) of the wild-type protein. 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 Y682 + 687G). (B) Immunoblots of cell lysates and conditioned medium from Mock-, wt-APP_{695}- and APPΔICD-transfected cells demonstrating APP holoprotein (antibody 6E10) and actin expression in lysates and sAPPα (antibody 6E10) and sAPPβ (antibody 1A9) levels in conditioned medium. (C) As for (B) but using cells transfected with the APP cytosolic tyrosine mutants. The lines on blots indicate where the lanes on the same immunoblot have been re-ordered for presentation purposes. (D) DU145 cells stably transfected with either vector alone (Mock) or the indicated APP construct were cultured over a 7 day period in the presence of copper (150 μM). At 7 days cell viability was determined as described in the Section 2. Results are expressed relative to the viability of Mock-transfected cells cultured in the presence of copper at day 7 and are means ± S.D. (n = 3). ** Denotes significance at P = 0.01.

expressed in alternative cell lines (HEK or SH-SY5Y) the two constructs were secreted as the predicted fragments of approximately 110 kDa (Fig. 2B and C). We, therefore, cultured mock-transfected DU145 cells in pre-conditioned medium from the HEK or SH-SY5Y stable transfectants (see Section 2) in the absence or presence of copper. The results (Fig. 2D) show that the growth of cells was

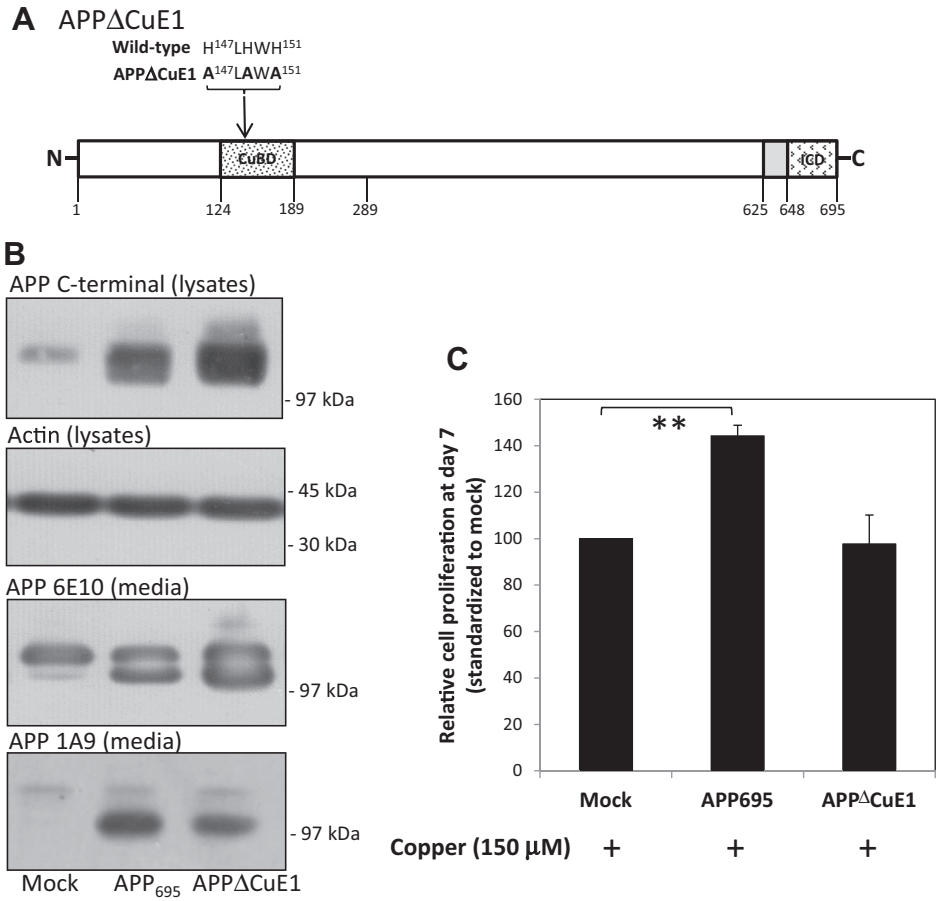

Fig. 4. The effect of the APP E1 copper binding domain on the growth of DU145 cells in the presence of copper. (A) The APPΔCuE1 construct possesses three histidine to alanine mutations at positions 147, 149 and 151 within the E1 copper binding domain (CuBD) of the protein. (B) Immunoblots of cell lysates and conditioned medium from Mock-, wt-APP_{695}- and APPΔCuE1-transfected cells demonstrating APP holoprotein and actin expression in lysates and sAPPα (antibody 6E10) and sAPPβ (antibody 1A9) levels in conditioned medium. (C) DU145 cells stably transfected with either vector alone (Mock) or the indicated APP construct were cultured over a 7 day period in the presence of copper (150 μM). At 7 days cell viability was determined as described in the Section 2. Results are expressed relative to the viability of Mock-transfected cells cultured in the presence of copper at day 7 and are means ± S.D. ($n = 3$). ** Denotes significance at $P = 0.01$.

unaffected by the presence of exogenous sAPPα or sAPPβ in the medium. Also of note is the fact that α- and β-secretase inhibitors had no effect on the growth of wt-APP-transfected DU145 cells in the presence of copper (data not shown). Collectively, these data indicate that the full-length form of APP is a prerequisite for the mitigation of copper-induced growth inhibition in DU145 cells.

3.4. Tyrosines 682 and 687 within the APP intracellular domain are pre-requisites for the mitigation of copper-induced growth inhibition

The fact that soluble forms of APP failed to mitigate copper-induced growth inhibition 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. 3A). In order to narrow down any potential involvement of the APP ICD even further, we also examined the role of two cytosolic tyrosine residues within this domain (tyrosines682 and 687) the phosphorylation of which might be linked to cell signalling. To this end, three further constructs were generated; APP Y682G, APP Y687G and APP Y682 + 687G (Fig. 3A). Following transfection of the constructs into DU145 cells, the holoprotein expression levels and generation of sAPPα were indistinguishable from those of wt-APP_{695} (Fig. 3B and C). However, no sAPPβ was generated from APPΔICD (Fig. 3B) which was probably indicative of the fact that the APP ICD is required for internalisation of the protein and subsequent β-secretase cleavage. Reduced levels of sAPPβ generation were also observed in the case of the tyrosine mutant constructs (Fig. 3C) indicating that these residues are specifically linked to the generation of this fragment. However, given our previous results showing that sAPPβ had no effect on DU145 cell growth in the presence of copper (Fig. 2) the reduced generation of this fragment from the cytosolic domain constructs is unlikely to have impacted on cell growth. The reduced growth of cells expressing these constructs in the presence of copper (relative to wt-APP-transfected cells) (Fig. 3D) was, therefore, most likely due to changes in the nature of the APP intracellular domain.

3.5. The APP E1 copper binding domain is a pre-requisite for the mitigation of copper-induced growth inhibition

Finally, we examined the role of the copper binding domain (CuBD) in the E1 extracellular domain of APP. We generated a construct (APPΔCuE1) in which three histidines were mutated to alanine in a key area of the protein associated with copper binding [18] (Fig. 4A). This construct was expressed and processed in DU145 cells in a manner identical to that of wild-type APP_{695} (Fig. 4B). However, APPΔCuE1 failed to mitigate copper-induced growth inhibition (Fig. 4C) indicating that the extracellular E1 CuBD, in addition to the cytosolic domain, of APP were prerequisites in this respect.

3.6. Summary

Our results indicate that APP is one element in a potential range of biochemical mechanisms by which cancer cells are able to proliferate efficiently despite the presence of elevated copper levels within the tumour microenvironment. DU145 cells, in particular, are able to increase their expression of the protein as a response to physiologically relevant concentrations of copper. At the molecular level, the ability of APP to mitigate copper-induced growth inhibition requires an intact E1 CuBD within the extracellular domain and probably involves a signalling pathway mediated by the phosphorylation of tyrosine residues within the cytosolic domain of the protein.

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