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Aminopeptidase O Contains a Functional Nucleolar Localization Signal and is Implicated in Vascular Biology

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Abstract We have identified a gene trap integration into Aminopeptidase O, the gene encoding a member of the M1 family of metalloproteases. Using the βgal reporter of the gene trap vector, we have revealed that at least some *ApO* isoforms are expressed predominantly in embryonic and adult blood vessels leading us to propose that *ApO* plays a role in vascular cell biology. The protein produced from an engineered *Gfp-ApO* fusion cDNA localises to the nucleolus in transfected COS7 cells. We confirm that indeed the APO protein contains a functional nucleolar localisation domain by demonstrating that GFP-APO fusion proteins that lack the predicted nucleolar localisation signal are retained in the cytoplasm. We report the existence of multiple alternatively spliced *Apo* isoforms that differ with respect to the presence of exons encoding important functional domains. Alternative splicing predictably produces protein products with or without the catalytic domain and/or a nucleolar localisation signal and therefore likely represents an important mechanism in regulating the biological activity of APO that has been reported to cleave one of the peptides of the renin angiotensin pathway. J. Cell. Biochem. 103: 1171–1182, 2008.

Key words: aminopeptidase O; angiogenesis; gene trapping; nucleolar localisation signal

The vascular system consists of a massive network of vessels which serve to transport blood and nutrients to organs and tissues. Endothelial cells form the lining of the vessels and they are ideally positioned to monitor and respond to paracrine stimuli. Under normal physiological conditions the endothelium perform a vast range of functions involving highly regulated processes such as angiogenesis, maintenance of blood pressure and wound healing. The endothelium also plays a significant role in the progression of many diseases, like solid tumour growth, rheumatoid arthritis, and hypertension [Brooks et al., 1998]. A key component of many of these processes both under normal physiology and pathological conditions are the proteases that are able to degrade extracellular matrix and control cellular processes such as cell cycle and proliferation [Roy et al., 2006].

The M1 aminopeptidases are a family of metallo zinc enzymes with a conserved catalytic domain (HEXXHX-E18) that catalyse the sequential removal of amino acids from the N-terminus of proteins or peptides [Taylor, 1993]. To date 13 mammalian M1 aminopeptidases have been described (www.uniovi.es/degradome) and demonstrated to be involved in a wide variety of biological processes involving angiogenesis, blood pressure regulation, and inflammatory immunological responses [Wright et al., 1990; Haeggstrom, 2004; Sato, 2004]. The membrane bound M1 Aminopeptidase N (APN/CD13), Aminopeptidase A (APA),

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and cytoplasmic puromycin insensitive leucyl aminopeptidase (PILSAP) are involved in angiogenesis. APN, for example, is expressed in the surface of endothelial cells of new vessels and tumours but not on quiescent vessels [Fukasawa et al., 2006] and specific inhibition of APN with a monoclonal antibody, functional inhibitors (bestatin), and siRNA mediated interference prevented capillary network formation in endothelial cell in vitro assays [Pasqualini et al., 2000; Fukasawa et al., 2006]. PILSAP is induced in endothelial cells by vascular endothelial growth factor (VEGF) stimulation and expressed in ECs during angiogenesis in vivo where it may mediate its effect though cell cycle pathways [Schomburg et al., 2000; Miyashita et al., 2002; Yamazaki et al., 2004]. APA has been shown to be involved in angiogenesis [Marchio et al., 2004] and the maintenance of blood pressure regulation via the renin angiotensin system (RAS) where it is responsible for the conversion of angiotensin II into angiotensin III [Mizutani et al., 1987; Ahmad and Ward, 1990; Mitsui et al., 2003]. Further evidence for APA involvement with blood pressure regulation has come from studies during pregnancy and the clinical hypertensive disorder preeclampsia [Mizutani et al., 1995; Mizutani and Tomoda, 1996]. Additionally, clinical studies during pregnancy and preeclampsia on the M1 aminopeptidase placental leucine aminopeptidase (PLAP) also implicate its involvement with blood pressure maintenance [Tsujimoto et al., 1992; Matsumoto et al., 2000]. More recently, it was shown that aminopeptidase O (APO) can cleave angiotensin III to generate angiotensin IV suggesting that this aminopeptidase may also belong to this important group of enzymes involved in vascular function [Diaz-Perales et al., 2005]. We provide further support for this hypothesis by describing the characterisation of a gene trap integration (GT411) into the *ApO* gene showing that *ApO* is expressed in the vasculature of the developing embryo and in the adult.

The distinct subcellular localisation of aminopeptidases that have been implicated in angiogenesis suggest distinct mechanisms of action. APN, for example, is a type II membrane ectoenzyme that has only nine amino acids in its intracellular domain and it presumably cleaves amino acids from the N-terminus of proteins in the extracellular space. It's exclusive expression on the endothelial cells of new vessels, but not of normal vasculature supports its involvement in angiogenesis via the modification of the extracellular matrix [Fukasawa et al., 2006]. PILSAP, on the other hand is a cytoplasmic aminopeptidase that is expressed in endothelial cells at the site of angiogenesis in vivo and is thought to act by mediating cell cycle progression [Miyashita et al., 2002; Yamazaki et al., 2004]. We provide experimental evidence to show that the APO protein contains a functional nucleolar localisation signal and that alternative splicing might play a role in the regulation of its biological activity.

MATERIALS AND METHODS

Gene Trapping

We used a directed gene trap approach to screen for gene trap integrations into genes that were expressed in ES-derived beating cardiomyocytes and associated cells [Forrester et al., 1996; Stanford et al., 1998; Hirashima et al., 2004]. E14 ES cells were maintained in the presence of LIF as described [Jackson et al., 2002] and electroporated with a mixture of 50 μg each pGT frame 0, 1, and 2 (provided by Dr. W. Skarnes) that had been linearised with Hind III. Neomycyin resistant colonies were selected in 200 μg genticin/G418 per ml for 10 days and resistant colonies were then replicated. One aliquot was frozen and the others were treated with or without retinoic acid (RA) (10^{-6} M) for 48 h as described [Forrester et al., 1996]. Clones in which the reporter gene (β-galactosidase (βgal)) was repressed in the presence of RA were selected for subsequent cardiomyocyte differentiation in vitro. Embryoid bodies (EBs) were generated in hanging drops (300 cells/10 μl drop) for 2 days in the presence of LIF then harvested and grown in suspension in the absence of LIF for a further 5 days. They were then plated onto gelatinised 24-well tissue culture wells (2–3 EBs per well) and recorded on video for the presence of beating regions over the following 14 days. Cells were then fixed and stained for βgal activity as described [Forrester et al., 1996].

Endothelial Cell Culture

The murine cell endothelial cell line (MCEC), a gift from Justin Mason, was cultured as previously described [Lidington et al., 2002] and the human umbilical vein endothelial cells (HUVEC) cell line was cultured in EGM

supplemented with bullet kit (Cambrex) [Rajesh et al., 2005].

Mouse Breeding and Genotyping

The GT411 gene trap cell line was transmitted through the germline by injection of C57/Bl6 blastocysts. Chimaeric males were backcrossed for three generations to wild type MF1 out bred females before intercrossing heterozygous animals. Adult animals were genotyped by staining an ear punch biopsy or the yolk sac for βgal activity. Homozygous ($ApO^{Gt411For/Gt411For}$) and heterozygous ($ApO^{Gt411For/+}$) animals for the gene trap integration were distinguished by the speed of appearance and the intensity of the staining reaction. The reliability of this genotyping method was confirmed by breeding analysis (data not shown).

RNA Isolation and Northern Blot Analysis

Tissues were snap frozen in liquid nitrogen, homogenised (Polytron) in Trizol (Invitrogen) and HUVEC and MCEC cell lines were lysed in Trizol then total RNA was extracted according to the manufacturers protocol. We obtained RNA that was derived from the primary human pulmonary artery smooth muscle cell line from TCS Cellworks Ltd. An Oligotex mRNA extraction kit (Quiagen) was used to isolate mRNA. For Northern blot analysis 3 μg of mRNA was electrophoresed in a 1% agarose-2.2 formaldehyde gel at 90 V for 3 h. The gel was washed in distilled water, placed in 0.05 M sodium hydroxide/1.5 M sodium chloride for 30 min then neutralised in 0.5 M Tris/HCl pH 7.4/1.5 M sodium chloride for 20 min. RNA was transferred to Hybond N+ (Amersham/Pharmacia) in 10× SSC overnight then baked at 80°C for 2 h. Hybridisation was carried out at 42°C overnight in Ambion Ultrahyb with ^{32}P-dCTP labelled probes (Roche High prime kit) then washed under high stringency conditions (0.1% SSC/0.1% SDS at 55°C) before exposure to X-ray film at −70°C with an intensifying screen.

Race-PCR

Rapid amplification of cDNA ends (RACE) was carried out as described previously [Townley et al., 1997]. Primer LZ1 (Table I), complementary to *lac* Z, was used to generate first strand cDNA from total RNA isolated from ES cells carrying the GT411 gene trap integration. The first round PCR reaction used the anchor primer 1 (Table I) paired with the nested primer LZ2. A second round of PCR used a second anchor primer 2 with the paired primer, En2 which was specific to the exonic region of the splice acceptor of the gene trap vector. The PCR conditions were 30 cycles of 94°C for 1.5 min, 60°C for 1.5 min then 72°C for 1.5 min. Further 5′ RACE was carried out on mRNA isolated from day 11.5 post-coitus wild type mouse embryos. Using a Gene Racer kit (Invitrogen) cDNA was generated as manufacturers instructions and the first round of PCR was carried out with GT411/3 (Table I) and AP1 (Invitrogen) and then nested with GT411/2 (Table I) and AP2 under the following 'touch down' PCR conditions using Thermozyme Taq (Invitrogen). Samples were incubated at

TABLE I. Primers Used in RT-PCR Analysis

2	CATTTCGCCACCTCGGAGCCTACTTG
3	CTGAGGGCTCAGCGTCTTCTGCTCTA
11	TGTCCATGTGTTCCTTCACCAGC
14	CAGGTGTTAAGACCCAACAAAGAA
15	ATCCTGGGAAAGAATCAGCTGC
21	GCAGCTGCTGTGCCTCAGCCCAAAATA
23	GGCAGCTGATTCTTTCCCAGGAT
24	AAAGCAAGACGATCTGGTCTTGGAG
26	TAACCTGCAGCTCCGGTGTCAAGCAG
36	CAGCGGACGCACTCTGGAACCTTGCA
55	TATTTTGGGCTGAGGCACAGCAGCTG
57	TTAGAACAGCATTTCAGTCACCACCTG
Fib1	GAATTCATGAAGCCAGGTTTCAGCCCCCGTG
Fib 2	GGTACCTCAGTTCTTCACCTTGGGAGGTG
LZ1	TAATGGGATAGGTTACG
En2	TGCTCTGTCAGGTACCTGTTG
Anchor P1	GGTTGTGAGCTCTTCTAGATGGTTTTTTTTTTTTTTTTT
Anchor P2	GGTTGTGAGCTCTTCTAGATGG
hum3	GATGCAGGTGTTAAGACCCAGTAA
hum2	AGATGCTCCAGAAGCAAGACCAGCTG
hum4	GGGCTGAGGACCTATCCATCTGCTC
SMAhum1	CCGATAGAACATGGCATCATCAC
SMAhum2	CCATCAGGCAACTCGTAACTCTTC

94°C for 2 min then subjected to the following PCR: 5 cycles of 94°C for 15 s, 72°C for 30 s, and 72°C for 2 min, then 5 cycles of 94°C for 15 s, 70°C for 30 s, and 72°C for 2 min, then 25 cycles of 94°C for 15 s, 68°C for 30 s, and 72°C for 2 min. Products were cloned into the TA cloning vector (Invitrogen) and sequenced.

RT-PCR

First strand cDNA synthesis was performed on 250 μg mRNA using superscript RNase Reverse Transcriptase (Invitrogen). After cDNA synthesis the 25 μl reaction volume was brought to 50 μl with ddH_20. For each PCR reaction 1 μl of cDNA was used. The reaction was incubated for 2 min at 94°C followed by 30 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s.

ApO-GFP Construct Generation

To study the subcellular localisation of *ApO*, deletion constructs were generated by restriction enzyme PCR and cloned into the C2 eGFP vector (Clontech). A control fibrillarin-eGFP fusion cDNA was generated by RT-PCR and all clones were sequenced to confirm correct engineering.

In Situ Hybridisation

Embryos were dissected from CD1 mice, fixed in 4% PFA/PBS and dehydrated in increasing concentrations of methanol before storing at −20°C. Digoxigenin labelled antisense *ApO* riboprobes were synthesised by linearisation of vector with *Eco*RI containing cDNA probe N1 (nucleotide 2,260–3,264) using the Dig riboprobe kit and T7 RNA polymerase (Roche). Whole mount in situ hybridisation of embryos with Digoxigenin labelled riboprobes was carried out as described [Wilkinson, 1992] and detected using NBT/BCIP.

RESULTS

Isolation of ES Cell Line Carrying the GT411 Gene Trap Integration

From a total of 229 G418 resistant colonies, 47 clones in which the reporter gene (β-galactosidase (βgal)) was repressed in the presence of RA were selected for subsequent cardiomyocyte differentiation in vitro. The GT411 ES cell line was one of 42 clones that showed βgal expression in the regions of beating cells and was selected for further analysis.

cDNA and Genomic Structure of *Apo*

5′RACE extension using primers complementary to the gene trap vector identified 206 base pairs (bp) of novel sequence that was used to screen expressed sequence tags (EST) databases. A contig of overlapping ESTs (AA560030, AA003012, and AA755402) was generated then additional 5′ RACE experiments were performed to extend this sequence using gene specific primers until a methionine residue was identified that had an upstream inframe stop codon. The translated sequence predicted a full length ORF encoding a 491 amino acid protein that was 72% similar to the human APO sequence [Diaz-Perales et al., 2005], and 95%, and 38% similar to the rat, and puffer fish, sequences, respectively (Fig. 1a, Supplementary S1, S2). APO contains a consensus sequence HEXXH(X)18E which is typical of the zinc aminopeptidase M1 family and is totally conserved in all species analysed. An armadillo repeat region is predicted between residues 710 and 823 and manual comparison of the APO protein sequence with known nuclear localisation signals (NLS) indicated the presence of a putative NLS between residues 694 and 70 (Fig. 1, Supplementary S1).

Alternative Splicing Generates Functionally Distinct APO Isoforms

5′RACE analysis revealed the presence of several *Apo* isoforms that were predictably produced by alternative splicing. To confirm this, RT-PCR and Northern blot analysis was performed and the intron/exon structure of the *Apo* gene was assembled from the published mouse (http://genome.ucsc.edu chromosome 13, 61300451–61635774) genomic and cDNA sequences (Fig. 1). We first noted that the *Apo* gene has two alternative start ATG sites encoded on exons 2 and 4. In RT-PCR experiments using primer pair 26/36, two bands of 460, and 1,393 bp were amplified (Fig. 1c lane 1). Cloning and sequencing of these bands revealed that these represented alternative splice forms. One of the transcripts is predicted to use the initiation codon present at position 332 that has a well-conserved Kozak [2002] resulting in the first six amino acids of the translated protein to be MPMPAS (Supplementary Fig. S1). The other transcript is predicted to use the initiation codon at position 1 (MDIKL) as it was proceeded by an

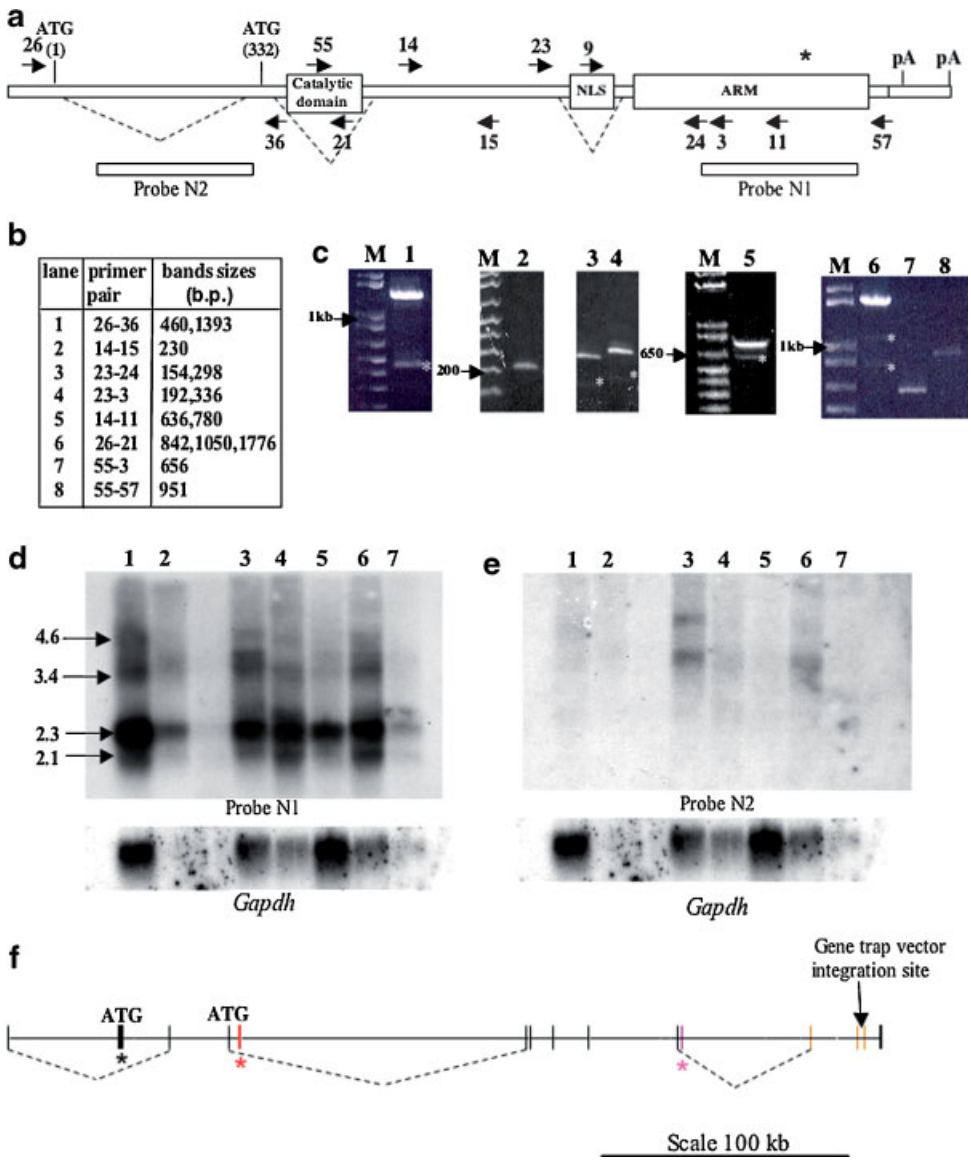

Fig. 1. **a**: Schematic diagram (not to scale) of *Apo* cDNA showing position of ATG start sites, polyA regions, regions encoding functional domains, primers used in RT-PCR analysis (**b**,**c**) and probes used in Northern blots (**d**,**e**). The site of the gene trap vector integration is marked with an asterisk. Northern blot of RNA isolated from one-month-old mouse heart (**lane 1**), brain (**lane 2**), lung (**lane 3**), liver (**lane 4**), skeletal muscle (**lane 5**), kidney (**lane 6**), ovary (**lane 7**) with probe N1 (d) and N2 (e) and the same gels hybridised to a *Gapdh* loading control as indicated. (**f**) Intron/exon structure of the Apo locus showing exons encoding the catalytic domain (red), the nuclear localisation signal (pink) and the armadillo repeat region (orange). The intron into which the gene trap vector has integrated is marked and asterisks mark the exons that we have shown to be alternatively spliced.

inframe stop in the upstream 5′ untranslated region (Supplementary Fig. S1).

Full length transcripts using primers specific to the ATG codons and primer 57 generated amplicons that lacked the 297 bp exon encoding the catalytic domain (supported by ESTs AK027581, and NM_032823), however using overlapping primers 26–21, and 55–57 specific to the catalytic domain we were able to generate amplicons that contained this domain (supported by ESTs AK057450, BU512973, and BY708307) (Fig. 1c, lanes 6–8). Sequencing of 5′RACE and RT-PCR products also revealed isoforms that predictably encode proteins that lacked the nucleolar localisation domain. RT-PCR analysis using primers pairs 23/24, 23/3, and 14/11 (Fig. 1c, lanes 3–5) amplified two bands with a size difference of 144 bp.

Sequencing confirmed this to be the 144 bp exon that encodes the nucleolar localisation signal (NuLS). It was interesting to note that only a single band was generated when PCR primer 55 was used in conjunction with primers 3 and 57. Primer 55 lies within the alternatively spliced exon that encodes the catalytic domain and we confirmed by sequencing that this unique PCR product contained the NuLS. We can therefore speculate that the NuLS and the catalytic domains are always encoded within the same transcript and that transcripts encoding only one of these domains do not exist at detectable levels. This apparently coordinated alternative splicing might represent a mechanism that ensures correct subcellular localisation of enzymatic activity.

Northern blot analysis of RNA isolated from a range of adult tissues confirmed the existence of multiple *Apo* isoforms and indicated that some of these isoforms were tissue specific. Using a C-terminal probe (N1) predominant transcripts of 2.2 and 2.5 kilobases (kb) were detected in all tissues tested and additional, less abundant, larger transcripts of 3.4 and 4.6 kb were observed in heart, lung, liver, and kidney (Fig. 1d). The same filter was stripped and hybridised with a probe (N2) that was specific to the 5′ alternatively spliced exon, and, as predicted, the predominant shorter 2.2 and 2.5 kb transcripts were not detected using this probe (Fig. 1e).

Genomic Mapping and Structure

The mouse *ApO* gene was mapped to mouse chromosome 13 C1 by fluorescent in situ hybridisation (FISH) using a *Apo*-PAC clone (583C16). The human homolog was mapped on to chromosome 9q22 using the Gene-bridge 4 human/hamster radiation hybrid panel [Gyapay et al., 1996] a region syntenic to mouse chromosome 13 C1 (data not shown). This experimental mapping was confirmed by analysis of the now published mouse (http://genome.ucsc.edu chromosome 13, 61300451–61635774) and human (http://genome.ucsc.edu chromosome 9, 94568538–94928995) genomic sequences. The region of chromosome 9q22 has been reported to be deleted certain cancer types including basal cell and oesophageal carcinomas [Lichun et al., 2004], bladder [Simoneau et al., 2000; Obermann et al., 2004] and ovarian cancer [Byrom et al., 2004].

Subcellullar Localisation

Xgal staining of the GT411 ES cells line indicated that the APO-βGAL fusion protein localised in distinct punctate regions within the cell nucleus (data not shown). To further characterise the subcellular localisation we transfected an N terminal GFP-tagged full length *ApO* construct into COS 7 cells and demonstrated that this protein also localised to punctate regions of the nucleus and specifically to the nucleolus (Fig. 2a). To test whether the

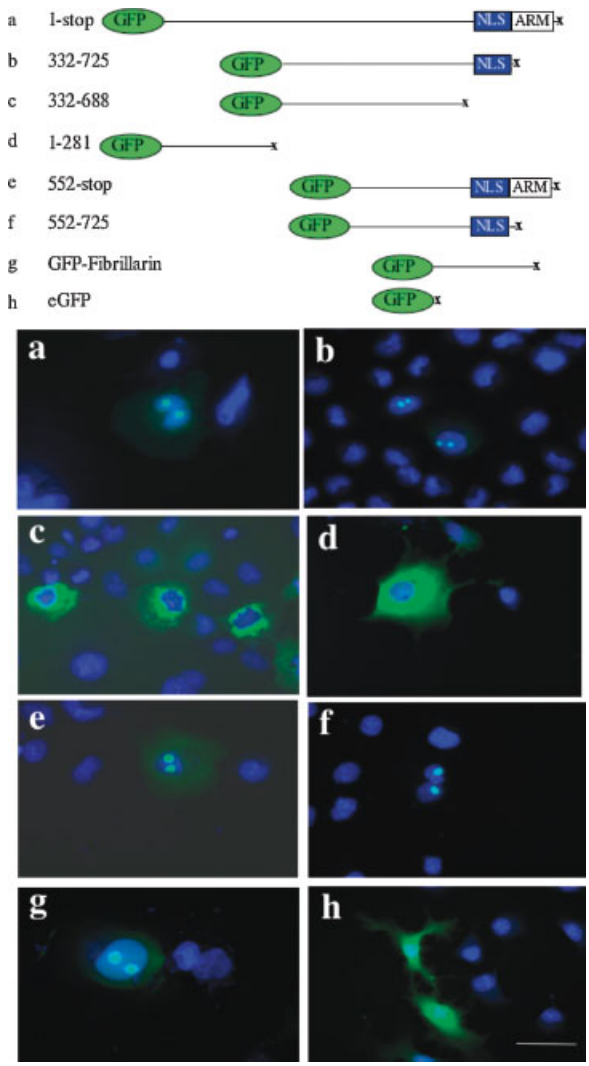

Fig. 2. DAPI-stained COS 7 cells transfected with full length *Apo* (**a**), and various truncated forms of *ApO* fused to *Gfp* as indicated (**b**–**f**). Numbers refer to amino acid position within the APO protein (see Supplementary Fig. S1). A *Fibrillarin-Gfp* fusion cDNA (**g**) and a *Gfp* alone (**h**) are shown as controls for nucleolar and cytoplasmic localization, respectively. Photomicrographs show representative fields of the subcellular localisation patterns that were observed in the majority of transfected cells in repeated transfections (scale bar 10 μm).

predicted nucleolar localisation signal (NuLS) (RRPRKRKRGKR) could guide the APO protein to the nucleolus, a range of eGFP constructs were generated, and transfected into COS7 cells (Fig. 2b–f). GFP was detected in punctate regions of the nucleus when constructs that contained the NuLS were used (Fig. 2a,b,e, and f) whereas GFP remained in the cytoplasm when constructs that did not contain the NuLS were used (Fig. 2c,d, and h). The subcellular localization pattern was identical to that observed using a GFP-tagged fibrillarin control (Fig. 2g) a gene known to localise to the nucleoli [Newton et al., 2003].

Apo-βgal Reporter Gene is Expressed in the Vasculature of Embryos and Adults

We predict that the βgal reporter of the gene trap vector marks the transcriptional activity of the *ApO* locus in mice carrying the GT411 gene trap integration. We detected βgal expression in the dorsal aorta of day 8.5 pc embryos and in the capillary plexus of the head and throughout the vasculature of the body at day 9.5 pc, most notably in the intersomitic vessels (Fig. 3a,b). To show that the reporter gene reflects the expression of the endogenous gene we preformed whole mount in situ analysis using an *ApO* specific probe. Although the probe used showed a relatively high level of background staining we did show that *ApO* was expressed in a comparable manner to βgal and was indeed expressed in the intersomitic vessels of 9.5 days embryos (Fig. 3c,d). In adult tissue sections we show that the expression of βgal was also restricted to the vasculature of adult spleen and kidney (Fig. 3f,h) as well as liver, skeletal muscle, and brain (data not shown). Interestingly in the adult heart staining was observed in blood vessels and diffuse staining was also observed in the intercalated discs of cardiomyocytes (Fig. 3j). This observation is consistent with the fact that that the gene trap screen was designed to identify genes expressed in beating cardiomyocytes produced in vitro.

We next sought to determine in which cell type within the vasculature *ApO* was expressed. βgal activity was detected in both the endothelial cell layer and in the smooth muscle of paraffin sectioned adult aorta from $ApO^{Gt411For/Gt411For}$ mice (Fig. 4a) indicating that *ApO* was expressed throughout the vessel. To support this observation and to confirm expression of the endogenous *ApO* gene in both cell types we carried out RT-PCR analysis on RNA isolated from endothelial and smooth

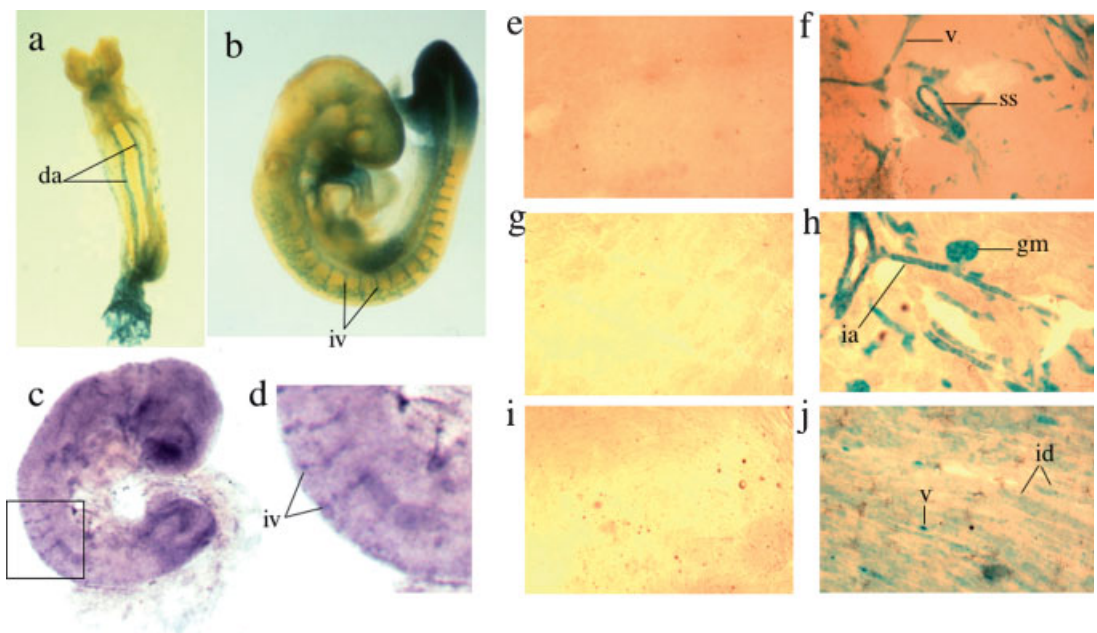

Fig. 3. βgal reporter gene expression in $ApO^{Gt411For/+}$ day 8.5 (**a**) and 9.5 (**b**) pc embryos and whole mount in situ analysis using the endogenous *ApO* probe (**c**) with enlarged image (**d**). Cryostat sections of adult spleen (**e**,**f**) kidney (**g**,**h**) and heart (**i**,**j**) from wild type (e.g., i) and $ApO^{Gt411For/+}$ (f,h,j) animals. Key: da, dorsal aorta, iv, intersomitic vessels, v, vein, ss, splenic sinusoid, ia, interlobular artery, gm, glomerulus, id, intercalated discs.

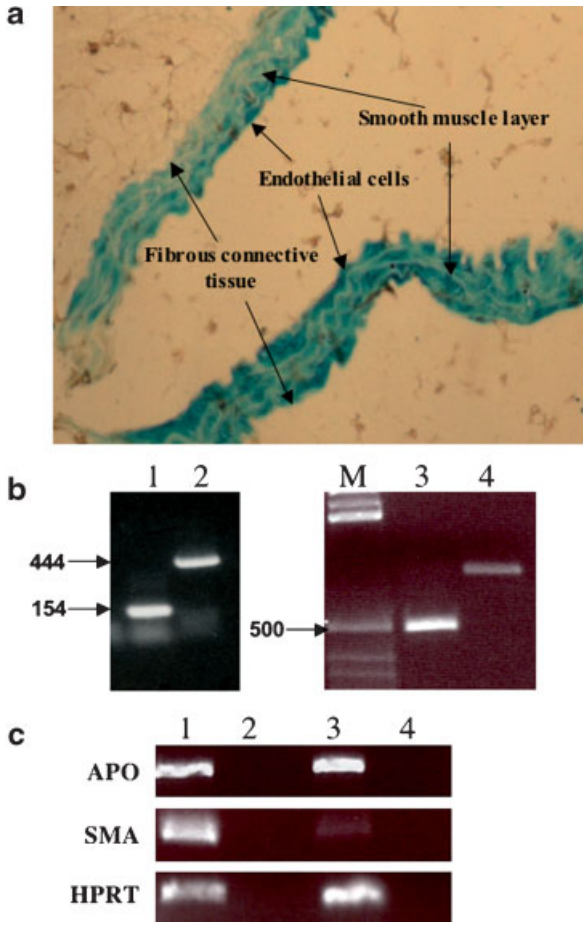

Fig. 4. **a**: βgal reporter gene expression in sections of adult aorta derived from $ApO^{Gt411For/Gt411For}$ mice. **b**: *ApO* expression analysis by RT-PCR of RNA isolated from murine endothelial cell line (MCEC) using primer pairs 9/3 (**lane 1**) and 9/11 (**lane 2**) and human vascular endothelial cells (HUVEC) using primer pairs hum3/hum2 (**lane 3**) and hum3/hum4 (**lane 4**). **c**: RT-PCR of RNA isolated from a human pulmonary artery smooth muscle primary cell line (lanes 1 and 2) and HUVECs (lanes 3 and 4) using the human *APO* primer pair hum3/hum4 and a primer pair (SMA1/2) designed to amplify smooth muscle actin (*SMA*) as a positive control for smooth muscle. Lanes 2 and 4 represent controls with no reverse transcriptase and primers complementary to *Hprt* are used as loading controls. All primers are listed in Table I and the position of the mouse *ApO* primers are shown in Figure 1.

muscle cell lines (Fig. 4b,c). Appropriately sized RT-PCR products were detected in all cases confirming that *ApO* is indeed expressed in both mouse and human endothelial cell lines as well as in a primary human pulmonary artery smooth muscle cell line (Fig. 4c).

Phenotypic Analyses

When matings were performed between mice that were heterozygous for the *ApO* gene trap integration homozygous animals were produced at the expected Mendelian ratio (25%) and showed no obvious deleterious phenotype. We used the aortic ring assay to test whether a subtle phenotype could be revealed by culturing aorta that had been dissected from adult animals in matrigel [Nicosia and Ottinetti, 1990]. We confirmed the identity of the endothelial cells in aortic sprouts using a fluorogenic labelled acetylated low density lipoprotein [Voyta et al., 1984] and showed that these cells also expressed βgal but we observed no significant difference in the sprouting of vessels from aorta dissected from wild type, heterozygous or homozygous adults (data not shown). We conclude that mice homozygous for the *ApO* gene trap integration do not have an overt phenotype which could either be due to the fact that the integration is in the 3′ end of the gene or that there is functional redundancy between *ApO* and other aminopeptidase encoding genes.

The Gene Trap Integration

Northern blot analysis showed that the integration of the gene trap vector results in the disruption of the *ApO* sequence downstream of the integration site. Using a probe that spans the integration site (N1, Fig. 1), the two predominant transcripts (2.2 and 2.5 kb) were detected in mRNA isolated from wild type and heterozygote but not homozygote embryos (Fig. 5). A transcript of 5 kb which is the

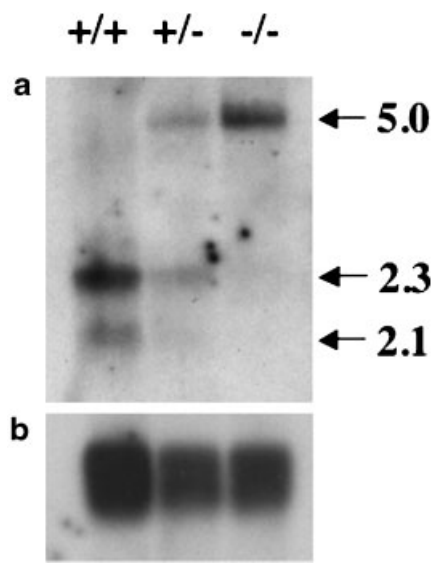

Fig. 5. Northern blot analysis RNA isolated from wild type ($ApO^{+/+}$), heterozygous ($ApO^{Gt411For/+}$) and homozygous ($ApO^{Gt411For/Gt411For}$) day 11.5 pc embryos using the N1 probe (**a**) which spans the gene trap integration site (see Fig. 1) and a *Gapdh* loading control (**b**).

expected size for the *ApO-βgeo* fusion, was detected in RNA isolated from heterozygote and homozygote but not wild type tissue as predicted. Furthermore, the intensity of this band in RNA isolated from heterozygote band was approximately half the intensity of the homozygote. We therefore conclude that no full length wild type transcript is produced in homozygous tissue and that we have no evidence for splicing around the gene trap vector to a produce a hypomorphic allele as has been reported in a number of gene trap integrations [Gasca et al., 1995; Voss et al., 1998]. However it is important to note that the integration of the gene trap vector has occurred near the 3′end of the gene and the resulting APO protein produced, although now fused to βgeo is only lacking 46 amino acids compared to the wild type protein. It is possible that this fusion protein could function as an aminopeptidase and could explain why no obvious phenotype has been observed in homozygous animals.

DISCUSSION

Using a gene trapping strategy designed to isolate genes involved in cardiovascular development we have cloned and characterised the murine orthologue of the human aminopeptidase O (*ApO*) [Diaz-Perales et al., 2005]. Using the βgal reporter we show that *ApO* is expressed in the vasculature of the developing embryo and continues to be expressed in the vascular tissues of the adult.

APO belongs to the M1 family of aminopeptidases which contain the HEXXH(X)18 motif and a central Zn^{2+} ion that are both essential for their enzymatic activity. To date 13 mammalian aminopeptidases of this family have been identified including both membrane bound and intracellular proteins (http://uniovi.es/degradome/) [Puente et al., 2003]. We noted that APO did not contain recognizable signal sequence or type II transmembrane domain indicating that it likely belonged to the cytoplasmic family of enzymes as previously suggested [Diaz-Perales et al., 2005]. In our study we have identified a putative nucleolar localisation signal (NuLS) in the APO protein sequence indicating it could be translocated to the nucleolus. Although this NuLS that was not identified using the classical bioinformatic protein search programmes we confirmed its functionality by showing that APO-βGAL and APO-GFP fusion proteins are transported to the nucleolus only when this domain is present in the protein. EST database searching and RT-PCR analysis identified alternatively spliced *Apo* transcripts predicted to encode proteins that do not contain the NuLS or the catalytic domain suggesting that alternative splicing might be one mechanism regulating subcellular localisation and biological activity of this protein.

It has been proposed that APO, APB, APB-like, and LTA4 hydrolase may have diverged from an common ancestor giving rise to the M1 ARM family of aminopeptidases that all contain an Armadillo repeat (ARM) structure [Diaz-Perales et al., 2005]. The ARM domain was first identified in the *Drosophila melanogaster* segment polarity gene Armadillo [Peifer et al., 1993] and has since been shown to be present in a number of other proteins including *Importin* and *Plakophilins* where it has been shown to bind to the nuclear localisation signals of other proteins, transporting them to the nucleus [Klymkowsky, 1999; Conti and Izaurralde, 2001]. Although no specific function of the ARM repeat has been attributed to the M1 aminopeptidases the ARM repeat structure is thought to allow protein–protein interactions and may therefore be involved in the trafficking of other proteins. It is interestingly to note that the ARM-containing LTA4 hydrolase is located in the nucleus in hyperplastic type II alveoloar epithelial cells and leaves the nucleus as these cells differentiate into type I-like nonproliferative cells [Brock et al., 2005] but this protein does not appear to have a NLS sequence. It is possible that nuclear entry of LTA4 hydrolase is mediated via the ARM domain the structure of which has indeed been confirmed by crystallography studies [Thunnissen et al., 2001]. We noted that the presence of the ARM domain in the NLS-containing APO fusion proteins had no obvious effect on its subcellular localisation but it will be interesting to test whether the ARM domain alone can influence entry into the nucleus.

We observed expression of *ApO* in the vasculature of all adult tissues analysed by βgal staining of animals carrying the gene trap integration and by Northern blot analysis of tissues isolated from wild type animals which is consistent with the published report that *Apo* is expressed in a range of different adult human tissues [Diaz-Perales et al., 2005].

The vascular-restricted expression profile reported here is particularly interesting because it has been shown that APO cleaves angiotensin III to generate angiotensin IV, a bioactive peptide of the renin angiotensin (RAS) pathway with multiple actions on diverse tissues including brain testis and heart. In addition, it has been reported that nuclear translocation of angiogenin and other angiogenic molecules is a critical step in the process of angiogenesis [Moroianu and Riordan, 1994]. We propose that APO could be involved in the regulation and/or stability of these proteins and that a further regulation of their activity might be related to the subcellular localization of APO.

Our data indicate that APO is expressed in both the endothelial cells and the smooth muscle cell layer of the vasculature and might add weight to the hypothesis that these two cell types are derived from a common progenitor. The origin of smooth muscle cells surrounding the vasculature has been proposed to be largely from the neural crest [Le Lievre and Le Douarin, 1975] and surrounding mesenchyme [Thayer et al., 1995]. There has been some indication from cell lineage tracing experiments in chick embryos that vascular endothelial cells can directly contribute to the SMC layers surrounding the developing vasculature [DeRuiter et al., 1997]. Moreover, evidence from in vitro experiments may support the possibility that there is a common progenitor of both endothelial and smooth muscle cells [Ema et al., 2003].

One explanation for the lack of an overt phenotype in the animals homozygous for the gene trap integration into *ApO* is the fact that that the integration has occurred in the last intron of the gene. This results in truncation of only 46 amino acids of the APO protein and, given that the catalytic domain and putative NLS would remain intact, it is possible that this protein retains all or partial function. We cannot however exclude the possibility that genetic redundancy could account for the absence of an overt phenotype during vascular development as suggested for APA null mice [Marchio et al., 2004]. Studies are under way to disrupt APO by conventional and conditional gene targeting to address this question. Interestingly, APA null mice show a severe impaired angiogenic response to oxygen-induced retinopathy and failed to sprout new blood vessels into subcutaneously implanted angiogenic factor containing sponges despite their lack of a developmental phenotype [Marchio et al., 2004]. We were unable to reveal a more subtle phenotype of our *ApO* mice using the aortic ring assay in vitro, but to date, have not performed more subtle phenotypic tests in vivo.

Drugs that target or inhibit the activity of aminopeptidases have been considered in cancer therapy. The identification of APO as a potential regulator of angiogenesis offers valuable prospects to inhibit tumour growth with therapeutic drugs directed to a vascular restricted protease.

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