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Apelin, the ligand for the endothelial G-protein-coupled receptor, APJ, is a potent angiogenic factor required for normal vascular development of the frog embryo

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Abstract

The peptide growth factor apelin is the high affinity ligand for the G-protein-coupled receptor APJ. During embryonic development of mouse and frog, APJ receptor is expressed at high levels in endothelial precursor cells and in nascent vascular structures. Characterization of *Xenopus* apelin shows that the sequence of the bioactive region of the peptide is perfectly conserved between frogs and mammals. Embryonic expression studies indicate that apelin is expressed in, or immediately adjacent to, a subset of the developing vascular structures, particularly the intersegmental vessels. Experimental inhibition of either apelin or APJ expression, using antisense morpholino oligos, results in elimination or disruption of intersegmental vessels in a majority of embryos. In gain of function experiments, apelin peptide is a potent angiogenic factor when tested using two in vivo angiogenesis assays, the frog embryo and the chicken chorioallantoic membrane. Furthermore, studies using the mouse brain microvascular cell line bEnd.3 show that apelin acts as a mitogenic, chemotactic and anti-apoptotic agent for endothelial cells in culture. Finally, we show that, similar to a number of other angiogenic factors, expression of the apelin gene is increased under conditions of hypoxia. Taken together, these studies indicate that apelin is required for normal vascular development in the frog embryo and has properties consistent with a role during normal and pathological angiogenesis.

Keywords: Angiogenesis; Apelin; APJ; *Xenopus*; CAM assay; Hypoxia; Endothelial mitogen

Introduction

The first blood vessels in the embryo are formed by a process called vasculogenesis, in which discrete vascular endothelial precursor cells (angioblasts) assemble into a tubular vascular plexus (Poole and Coffin, 1989; Cleaver and Krieg, 1998). All subsequent growth of blood vessels into avascular tissues is achieved through angiogenesis, which involves branching and outgrowth of vessels from the initial vascular network. In the frog embryo, the aortic arch vessels, posterior cardinal veins, the dorsal aorta and the vitelline veins form through a vasculogenic mechanism. The most prominent example of angiogenesis in the early embryo is the formation of the intersegmental (or intersomitic) vessels, which branch from the rudimentary posterior cardinal veins.

Numerous secreted growth factors play a regulatory role in the growth and maintenance of blood vessels. For example, vascular endothelial growth factor (VEGF-A) is a potent mitogen for angioblasts and endothelial cells and gene ablation experiments in mouse have demonstrated an absolute requirement for VEGF-A and its high affinity tyrosine kinase receptor VEGFR-2 (flk-1) for embryonic vascular development (Carmeliet et al., 1996; Ferrara et al., 1996; Shalaby et al., 1995). The VEGF signaling pathway also plays an important role during pathological growth of blood vessels. A large proportion of human tumors express high levels of VEGF, and this expression is directly related to their degree of vascularization and rate of

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overall growth (reviewed in Ferrara, 2002a). Conversely, reduction of VEGF signaling activity strongly correlates with inhibition of tumor growth (Kim et al., 1993; Ferrara and Alitalo, 1999). The endothelial receptor tyrosine kinase Tie-2 (VEGFR-1) and its high affinity ligands Angiopoietin-1 and 2 (Ang-1 and Ang-2) are also required for embryonic vascular development (Dumont et al., 1994; Sato et al., 1995; Suri et al., 1996), and the extent of vascularization of tumors has also been correlated with signaling via the Tie-2 pathway (Lin et al., 1997; Ahmad et al., 2001; Shim et al., 2001; Oliner et al., 2004). A number of other growth factor signaling pathways are capable of regulating endothelial cell proliferation and function. In particular, several G-protein-coupled receptor (GPCR) family members and their cognate ligands are potent regulators of blood vessel growth. Examples include, but are not limited to, stromal cell-derived factor-1 (SDF-1) signaling through the CXCR4 receptor family (Salvucci et al., 2004), sphingosine-1-phosphate (S1P) signaling via the S1P(1–5) receptor family (Kluk and Hla, 2002) and endocrine gland vascular endothelial growth factor (EG-VEGF) which binds to the PKR1 and PKR2 receptors (Le Couter et al., 2001; Lin et al., 2002).

The G-protein-coupled receptor, APJ (also named angiotensin II receptor-like 1—Agtrl1), was originally identified in a survey for angiotensin-receptor-related sequences in the human genome (O'Dowd et al., 1993). Subsequent experiments however demonstrated that APJ does not bind angiotensin and for a number of years the ligand required for APJ activity remained unidentified. During early development, APJ expression is largely restricted to the endothelial cells and endothelial precursor cells of the developing vasculature (Devic et al., 1996, 1999; Cleaver et al., 1997). In adult tissues, APJ transcripts are present in a broad range of organs and tissue types including lung, heart and kidney, and expression levels are particularly high in neural tissues (O'Carrol et al., 2000). Additional studies indicate that APJ is expressed on the surface of endothelial cells throughout the adult vasculature (Katugampola et al., 2001) suggesting a role for APJ-mediated signaling in maintenance or modulation of vascular function.

A high affinity ligand for APJ, named apelin (APJ endogenous ligand), has been isolated using biochemical techniques (Tatemoto et al., 1998). Apelin mRNA encodes a 77-amino-acid preproprotein that is proteolytically cleaved to yield bioactive peptides 36, 17 and 13 aa in size. Each of these peptides contains the extreme C-terminal region of the precursor protein, and all bioactivity is thought to reside in the terminal 13 aa fragment (Kawamata et al., 2001). There is evidence that post-translational processing of apelin into the different peptide isoforms is regulated in a tissue-specific manner (Kawamata et al., 2001), but it has not been established whether the various apelin isoforms possess distinct signaling properties or whether differential processing of the precursor peptide is used to regulate apelin activity. Since the identification of the apelin ligand, experiments have implicated apelin/APJ signaling in regulation of a number of physiological processes. For example, studies using different model systems suggest that apelin may regulate fluid uptake (Reaux et al., 2001; Taheri et al., 2002) and food uptake (Taheri et al., 2002; Sunter et al., 2003; O'Shea et al., 2003), modulate blood pressure (Katugampola et al., 2001; Tatemoto et al., 2001; Cheng et al., 2003; Ishida et al., 2004) and function as a powerful regulator of cardiac contractility (Szokodi et al., 2002; Chen et al., 2003; Berry et al., 2004). Still other studies show that apelin and APJ are expressed during formation of retinal vessels (Saint-Geniez et al., 2002, 2003), and a recent report demonstrates that apelin possesses angiogenic activity for a retinal endothelial cell line (Kasai et al., 2004).

In this report, we show that apelin is required for normal vascular patterning of the frog embryo. In addition, a variety of in vivo and in vitro assays demonstrate that apelin is a powerful stimulator of blood vessel growth and endothelial cell proliferation. We provide evidence that these angiogenic properties are independent of the VEGF signaling pathway. The properties of apelin are consistent with a larger role in regulation of blood vessel growth in the adult, under both normal and pathological conditions.

Materials and methods

Recombinant DNA and peptides

Recombinants containing the frog (*Xenopus*) apelin preproprotein sequences were identified in the expressed sequence tag (EST) database, GenBank accession number BE680255. The *Xenopus* APJ recombinant construction (also called X-msr) has been described previously (Devic et al., 1996; Cleaver et al., 1997). Digoxygenin-labeled RNA probes for in situ hybridization were prepared using a MegaScript kit (Ambion). Apelin peptide was synthesized to greater than 90% purity (Sigma-Genosys). The N-terminal glutamine of native apelin was modified to a pyroglutamic acid residue to avoid cyclization and poor yield during the manufacturing process. Mutant apelin peptide, in which the final 4 residues were altered to alanine, was also synthesized.

Embryology and microinjection

Xenopus embryos were staged according to Nieuwkoop and Faber (1994). For bead implantation experiments, Affi-gel blue beads (50–75 μm Bio-Rad) were soaked in apelin peptide, mutant apelin peptide, BSA or recombinant mouse VEGF-164 (R&D Systems) for 1 h on ice. Beads were implanted beneath the epidermis in the posterior lateral region of stage 24–26 *Xenopus* embryos. Embryos were cultured in 0.2× MMR until stage 34, at which time they were fixed in MEMPFA. Whole mount in situ hybridization was carried out as described previously (Harland, 1991). For apelin over-expression experiments, approximately 500 pg of apelin mRNA was injected into one cell embryos, together with 500 pg of EGFP mRNA as tracer. At the blastula stage, small clumps of cells were isolated and implanted into the flank of stage 24 recipient embryos. At stage 34, vascular structures were detected by in situ hybridization using digoxygenin-labeled Xl erg probe (Baltzinger et al., 1999).

Loss-of-function experiments were performed using antisense morpholino (MO) oligomers (Gene-Tools). Two antisense MOs overlapping the initiation AUG were designed to block translation from both pseudoallelic copies of the apelin transcript: apelin MO1 5′-GTGCCCAAAGTCTGAGATTCATGTT-3′, apelin MO2 5′-GATTCATGTTTCTTGTGGCTGAGTG-3′. A 5-base mismatch MO of apelin MO2 was called apelin MO2mut: 5′-GATTgATcTTTgTTGTGcCTcAGTG-3′. The mismatch nucleotides are shown in lower case. For APJ, an MO was targeted immediately upstream of the initiation AUG apj MO1: 5′-AAGGCTGTGTGGAAGCAATAGAAAG-3′. A 5-base mismatch control sequence for the apj MO1 was called apj MO1mut: 5′-AAGcCTcTGTGcAAcCAATAcAAAG-3′. MOs were injected (in 50 mM HEPES pH 8.0) into one cell of the two-cell embryo, and the embryos were allowed to develop until stage 35 at which time they were prepared for in situ hybridization analysis. Texas Red Dextran (10 ng; Molecular Probes) was coinjected with the MOs as a lineage tracer.

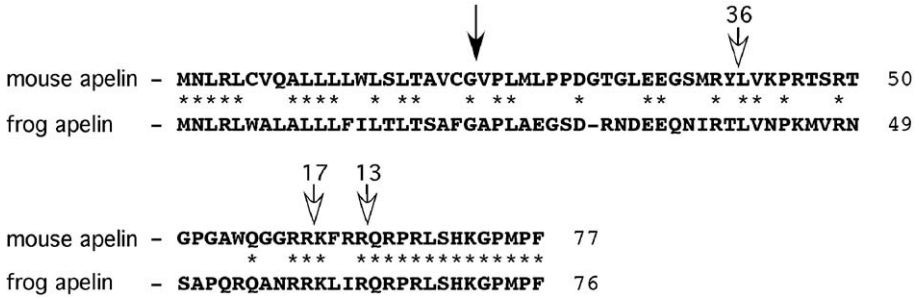


Fig. 1. Sequence alignment of mouse and *Xenopus* apelin preproproteins. Identical amino acids are indicated by asterisks. The vertical arrow indicates the position of cleavage of the leader sequence. The open arrows mark the positions of post-translational proteolytic cleavage to generate 36, 17 and 13-mer forms of apelin. Note that the sequence of the C-terminal bioactive region of the protein is perfectly conserved between frog and mouse. Mouse and *Xenopus* apelin preproprotein accession numbers are BG176301 and BE680255 respectively.

Chick chorioallantoic membrane (CAM) assay

Chicken eggs were incubated at 37°C in a humidified chamber. On day 10 of development, a window was made in the outer shell and a filter disk (3MM Whatman-8 mm diameter) carrying growth factor was placed onto the surface of the CAM. Filter disks were pretreated with 0.1% cortisone acetate solution to avoid inflammation of the CAM. After 3 days of incubation, the filter disks and the attached CAM were excised, washed with PBS and photographed for

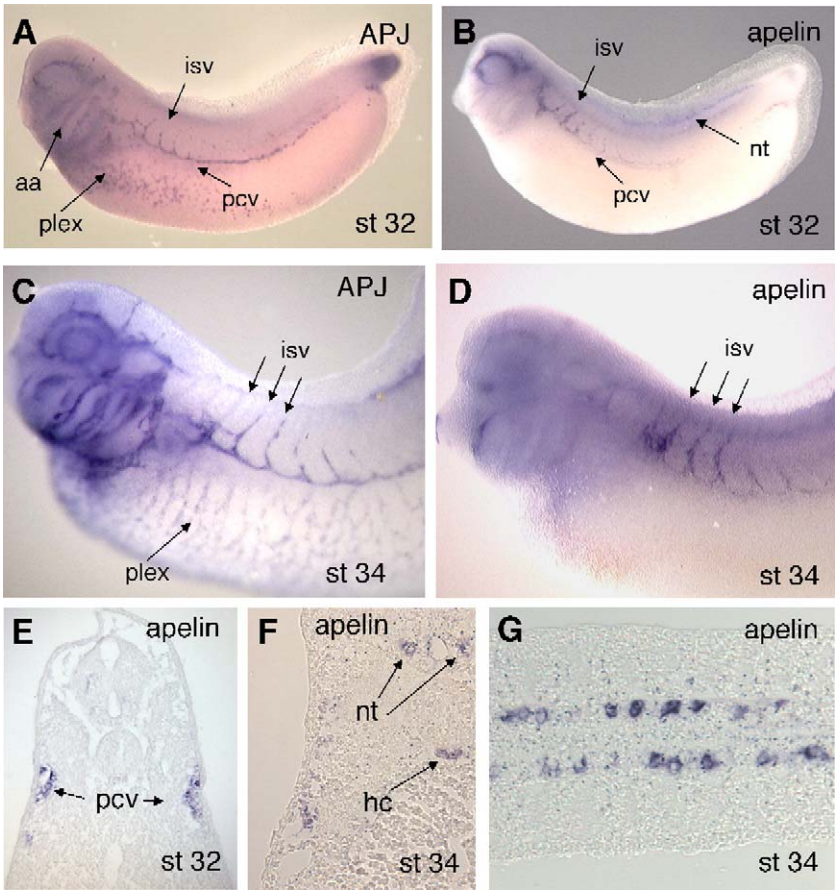


Fig. 2. In situ hybridization analysis of APJ and apelin expression during *Xenopus* development. (A) In the *Xenopus* embryo at the early tailbud stage (stage 32) APJ expression marks developing blood vessels throughout the embryo. The aortic arch region (aa) and the posterior cardinal vein (pcv), ventral vascular plexus (plex) and branching intersegmental vessels (isv) are indicated. The region of expression in the tailbud does not appear to be associated with vascular tissue. (B) At stage 32, apelin expression is detected in the anterior region of the posterior cardinal vein, the extending intersegmental vessels and the developing blood vessels surrounding the eye. Apelin expression is also detected in the ventral neural tube (nt). (C) At stage 35, APJ transcripts are detected throughout the developing vasculature, including the assembling vascular plexus in ventrolateral regions of the embryo and distinctly in the intersegmental vessels. (D) At stage 35, apelin expression is visible in the branching intersegmental vessels but expression in the vessels surrounding the eye is now absent. No apelin transcripts are detected in the plexus region. (E) Transverse section through anterior trunk region of stage 32 embryo stained for apelin transcripts. Note prominent expression in the posterior cardinal veins. (F) Transverse section through trunk of stage 34 embryo stained for apelin. In addition to posterior cardinal veins, expression is visible in the hypochord (hc) and in paired regions within the ventral neural tube (nt). (G) Longitudinal section through ventral neural tube of stage 34 embryo stained for apelin. Anterior is to the left. Note that expression is not continuous but appears to mark paired motor neurons (Saha et al., 1997) (H).

quantitative analysis. The number of vessel branch points (Wilting et al., 1991) for different treatments was counted in a blinded analysis.

Cell culture and analysis

Mouse brain microvascular endothelial cells (bEnd.3; ATCC) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (each 100 μg/ml; InVitrogen/Life Technologies). For proliferation studies, bEnd.3 cells plated at 70% confluence in 8-well culture slides were serum-starved for 24 h and then supplemented with growth factors for 24 h, after which BrdU (10 μM) was added to each well for 2 h. In some experiments, FGF receptor inhibitor (SU5402, 20 μM in DMSO) or VEGF receptor inhibitor SU1498 (25 μM in DMSO) was added to the culture media for 1 h prior to growth factor addition. BrdU analysis of cell proliferation was carried out as follows: after BrdU incubation, cells were air-dried, rehydrated for 3 min with PBS and then DNA was denatured at 37°C with 2 M HCl and neutralized with 2 × 5 min washes of 0.1 M borate buffer (pH 8.5). Cells were washed 3 × 5 min with PBS and blocked for 30 min in 1% normal goat serum/2% BSA. FITC-labeled anti-BrdU antibody (10 μg/ml; Sigma) in block was added for 1 h at RT. Cells were counter-stained with propidium iodide following several washes in PBS. Three random fields from 3 wells were photographed for determination of percentage BrdU labeling. Migration assays were performed using Trans-well migration chambers with 8 μm pore size (Becton Dickinson). bEnd.3 cells were plated in the upper chamber in the presence of 2% serum. Following cell attachment to the membrane and overnight serum starvation, growth factor was added to the bottom chamber for 6 h, after which cells that had migrated to the lower chamber were fixed and stained with DAPI (1 μg/ml) for 30 min and then counted. Apoptosis assays were carried out using the Promega Fluorescent TUNEL kit and the protocol recommended by the manufacturer. Hypoxia assays used primary rat cardiomyocytes and treatment for 4 h with 125 μM $CoCl_2$, as previously described (Chun et al., 2003).

Results

Developmental expression of apelin and APJ

The apelin receptor APJ is expressed at high levels in angioblasts and early vascular tissues of the *Xenopus* embryo (Devic et al., 1996; Cleaver et al., 1997). In order to investigate a possible role for apelin/APJ signaling during vascular development, we have identified clones encoding *Xenopus* apelin in the GenBank EST database. The deduced amino acid sequence of *Xenopus* preproapelin is aligned to the mouse protein in Fig. 1. Excluding the highly variable signal peptide (residues 1–22), the mouse and frog apelin proproteins are 51% identical overall, whereas the C-terminal 13 amino acids, comprising the bioactive region of apelin, are identical between the two species. The frog apelin precursor sequence retains the proteolytic cleavage sites found in the mouse preproprotein, suggesting that the previously characterized 36, 17 and 13 amino acid apelin isoforms will also be present in the frog.

Using in situ hybridization, we have compared expression of the APJ receptor and apelin ligand during *Xenopus* embryonic development. Initial vascular expression of APJ is detected in the frog embryo at approximately stage 28 in angioblasts that will contribute to the posterior cardinal veins and the aortic arch vessels (data not shown). At the early tailbud stage (stage 32), expression of APJ is visible in endothelial precursor cells in the aortic arch region, surrounding the eye, in the forming ventrolateral vascular plexus and in the developing cardinal vein (Fig. 2A). A second non-endothelial expression domain is also visible in the developing tail. At approximately stage 32, about 8 h after initial APJ receptor expression, apelin transcripts become visible in the anterior region of the posterior cardinal vein and more distinctly in vascular structures surrounding the developing eye (Fig. 2B). Apelin expression is also visible in the dorsal region of the embryo in posterior neural structures. Slightly later in development (stage 35), APJ continues to be expressed throughout developing vascular structures and staining remains visible in the ventral vascular plexus. Strong expression is also present in, or adjacent to, the endothelial cells of the first intersegmental vessels that are branching from the posterior cardinal vein (Fig. 2C). At this stage, apelin expression has been lost from vessels surrounding the eye but has become more pronounced in the intersegmental vessels (Fig. 2D). Apelin expression is not observed in the aortic arch region or in the ventral vascular plexus. Transverse sections through a tailbud embryo show that apelin transcripts are located in the endothelial cells of the posterior cardinal veins (Fig. 2E). This tissue localization is unambiguous since there are no smooth muscle cells associated with blood vessels at this stage of *Xenopus* development. Slightly later (stage 34), apelin transcripts are detected in the hypochord, a transient embryonic structure located immediately ventral to the notochord, and in paired regions within the ventral neural tube (Fig. 2F). Longitudinal sections show that apelin is expressed in individual cells on either side of the midline of the neural tube (Fig. 2G), a pattern that is consistent with the location of developing motor neurons (Saha et al., 1997). Based on these expression patterns, we conclude that apelin is expressed, either in immediate proximity to, or within, a subset of the endothelial cells expressing the APJ receptor. We also observe several expression domains of apelin, such as hypochord and neural tissues that are not in immediate proximity to APJ expressing cells. Note also that APJ is expressed in the ventral vascular plexus and the extreme tip of the developing tail, two domains that are not adjacent to regions of apelin expression.

Apelin stimulates angiogenic growth in the frog embryo

The embryonic expression patterns of apelin and APJ suggest that apelin may function to regulate vascular growth or maintenance, at least within certain regions of the embryo. We have tested this idea directly by implanting beads carrying apelin peptide into the frog embryo and then assaying for alterations in vascular patterning and growth. In these experiments, agarose beads were soaked with different concentrations of synthetic apelin(13) peptide, or mutant apelin(13) peptide (in which the last 4 residues were altered to alanine) and then inserted immediately below the epidermis in the ventral posterior region of the frog embryo. This region was selected because it is normally free of major vascular structures. The bead implantation was carried out at stage 24–26, prior to appearance of endothelial precursor cells in the embryo, and was assayed by in situ hybridization with Xlerg

(erg) probe at stage 34, by which time a well-established vascular network is normally present. Erg is a member of the ets family of transcription factors and serves as an excellent endothelial-specific marker in the frog embryo (Baltzinger et al., 1999). As shown in Figs. 3A–C, the presence of a localized source of apelin in the embryo stimulated a prominent outgrowth of endothelial cells from the region of the posterior cardinal vein towards the apelin bead. The effect was highly reproducible using a range of concentrations of apelin peptide (Table 1). Negative control experiments using beads soaked in mutant apelin(13) never produced alterations to the vascular structure (Fig. 3F and Table 1). Ectopic vascular structures were also observed when cells injected with mRNA encoding apelin preproprotein were implanted into the flank region of host tadpoles (Figs. 3D, E and Table 1). This result demonstrates that embryonic cells have the potential to process the apelin precursor protein and to secrete apelin in a bioactive form. In positive control experiments, agarose beads carrying VEGF (165) stimulated outgrowth of endothelial cells towards the source of VEGF (Fig. 3G and Table 1), similar to those observed with apelin. In view of the importance of VEGF in regulation of vascular growth, we considered the possibility that apelin might function by locally upregulating expression of VEGF in the embryo. If this is the case, then VEGF in the

Table 1
Stimulation of angiogenic growth in *Xenopus* embryo bead or cell implant experiments

Concentration of factor on bead	Number of embryos showing phenotype	Percentage phenotype
1 mg/ml apelin	25/28	89
0.5 mg/ml apelin	8/9	89
0.25 mg/ml apelin	10/11	91
0.1 mg/ml apelin	7/7	100
1 mg/ml BSA	1/12	8
0.25 mg/ml mutant apelin	0/10	0
0.25 mg/ml VEGF	8/16	50
apelin expressing cell implants	12/12	100
EGFP expressing cell implants	0/9	0

Beads were implanted at stages 24–26 and cells at stage 24. At stages 34–35, embryos were assayed for the presence of vascular tissue by in situ hybridization using probe directed against transcripts for the endothelial marker sequence erg.

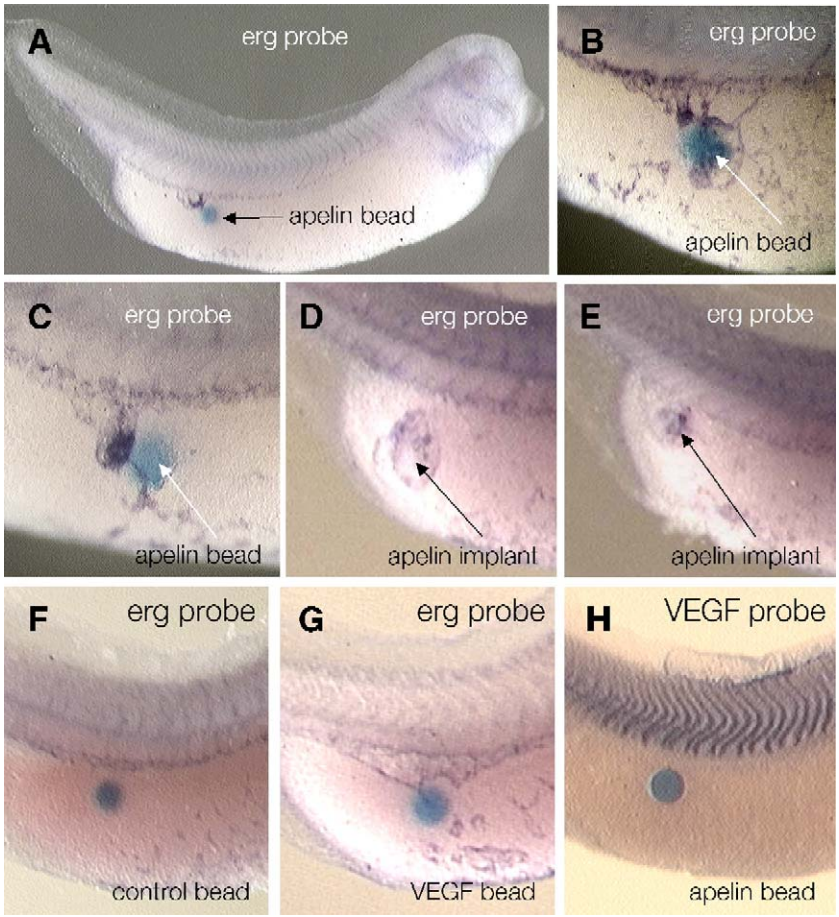


Fig. 3. Apelin is an angiogenic factor in the frog embryo. Porous beads treated with apelin, control peptide or VEGF, or cells expressing apelin protein, were implanted into frog embryos at stages 24–26, and vascular structure was assayed by in situ hybridization using the vascular-specific erg marker at stage 34. (A) Whole frog embryo showing location of apelin-soaked bead (arrow) and outgrowth of vascular tissue towards bead. (B, C) Enlarged views of vascular growth in the vicinity of the apelin bead. (D, E) Ectopic vascular cells in the vicinity of cell implants expressing apelin. Arrows indicate the position of the cell implants. (F) A bead soaked with mutated apelin peptide produces no ectopic marker expression. (G) A bead treated with VEGF(165) also produces vascular growth in the *Xenopus* embryo. (H) In situ hybridization analysis for VEGF transcripts shows that VEGF expression is not detected in the vicinity of the apelin bead 24 h after implantation but is detected within the somites as previously reported (Cleaver et al., 1997).

vicinity of the bead cells would be responsible for the endothelial cell outgrowth documented in Table 1. To test this possibility, we have assayed for VEGF expression around the apelin bead, using in situ hybridization. In this experiment we assayed for VEGF transcripts every 2 h (from 2 to 24 h) after implantation of the bead. Although VEGF transcripts were clearly detected in the somitic region of the embryo as previously reported (Cleaver et al., 1997), no VEGF expression was detected in the immediate vicinity of the apelin bead at any time examined (Fig. 3H). Although preliminary, this result suggests that apelin is not acting through upregulation of VEGF expression.

Inhibition of apelin/APJ signaling using morpholino oligomers alters vascular patterning in the frog embryo

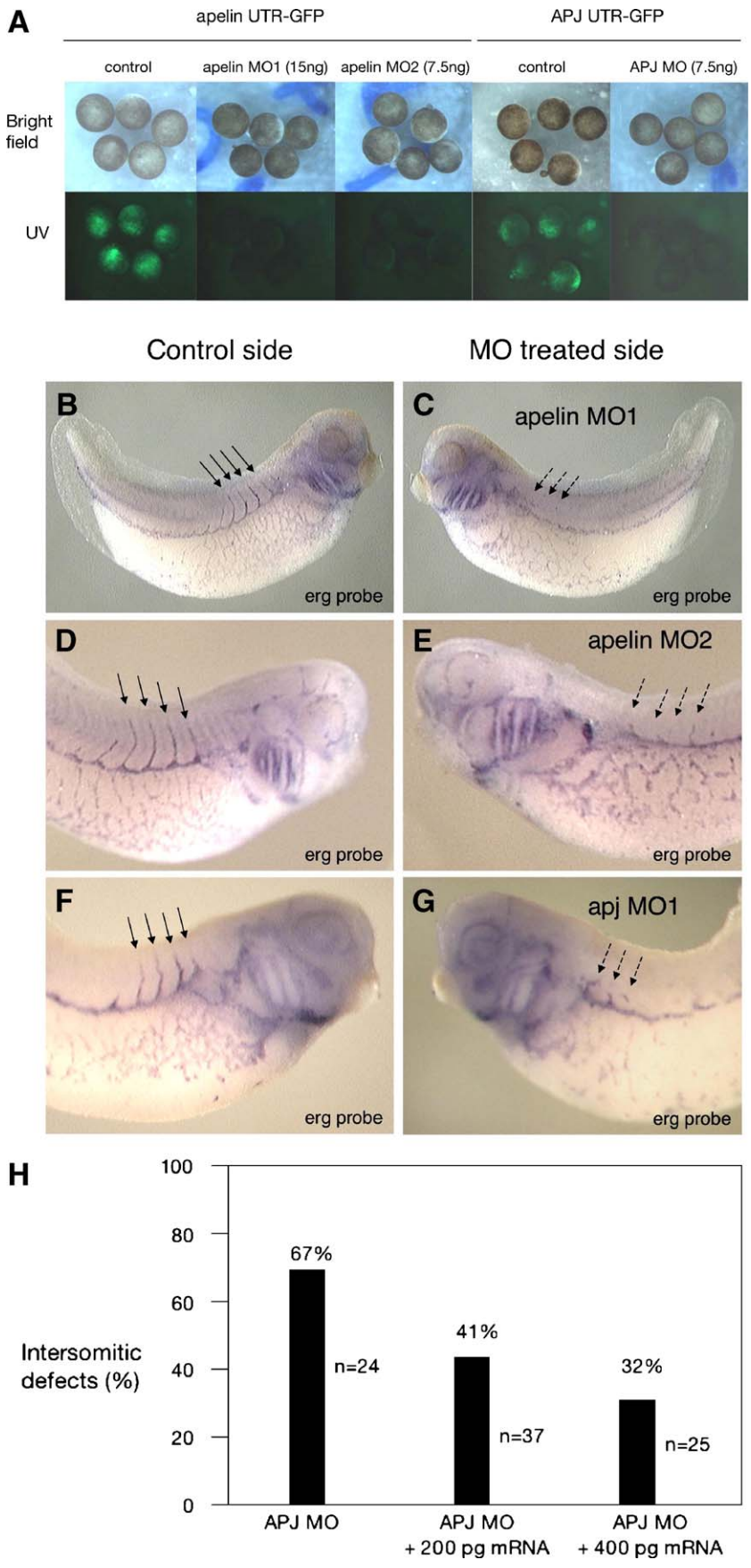
To investigate whether apelin signaling is required for embryonic vascular development, we have used morpholino oligomers (MOs) to translationally inhibit the expression of apelin and APJ. Two different MOs targeting apelin transcripts, apelin MO1 and apelin MO2, were used in these studies. Both MOs overlapped the initiation AUG, and both were shown to inhibit translation of apelin mRNA in the frog embryo, using methods previously described (Small et al., 2005, Fig. 4A). To carry out the loss of function studies, 15 ng of apelin MO1 was injected into one cell of the two-cell embryo. The uninjected side of the embryo acts as a stage-matched control. At the tailbud stage of development, vascular structures in the embryo were visualized by in situ hybridization for erg transcripts. As shown in Fig. 4, inhibition of apelin expression using apelin MO1 generated a distinct alteration in vascular pattern on the manipulated side of the embryo—specifically the reduction or loss of intersegmental vessels (compare Figs. 4B and C). Quantitation of the MO1 knockdown results showed that 67% of embryos (51/76) exhibited distinct reduction in intersegmental vessel number on the injected side of the embryo. We required that at least 3 intersegmental vessels be completely absent before an embryo was scored as reduced. To confirm these results with a different MO sequence, 7.5 ng of apelin MO2 was injected into one cell of the two-cell embryo and the resulting vascular pattern was assayed as described above. Using MO2, 63% of injected embryos (40/64) showed a distinct reduction in intersegmental vessel number (compare Figs. 4D and E). A control MO containing 5-base mismatches relative to apelin MO2 was injected at 15 ng per embryos (i.e. twice the dose used for apelin MO2 experiments), and subsequent analysis of vascular patterning showed that only 5% of embryos showed any reduction in intersegmental vessel number (2/38—data not shown).

In order to determine whether loss of receptor showed the same phenotype as loss of ligand, we used another MO (APJ MO1) to inhibit expression of the APJ receptor. This MO was demonstrated to inhibit APJ mRNA expression in control experiments (Fig. 4A). Injection of 7.5 ng of APJ MO1 into one cell of the two-cell embryo resulted in significant vascular patterning defects when assayed at the tailbud stage of development. As with the apelin knockdown experiments, the most prominent defect was a reduction or loss of intersegmental vessels on the injected side of the embryo (Figs. 4F, G). Quantitation showed that 71% (34/48) of APJ-MO1-injected embryos exhibited a significant reduction in the number of intersegmental vessels. Injection of a mismatched APJ control MO, at twice the experimental dose (15 ng), generated intersegmental defects in only 14% of embryos (8/58—data not shown). In some experiments, we also observed an apparent reduction in endothelial cell number and disorganization of structure in the region of the ventral plexus (see for example Fig. 4G), but this was only observed in a small proportion of cases. Finally, we demonstrate that coinjection of mRNA encoding the APJ receptor was able to partially rescue the vascular defects generated with APJ MO1 (Fig. 4H). Overall, these inhibition experiments suggest a role for apelin/APJ signaling in regulation of vascular patterning in the *Xenopus* embryo, particularly in the growth of the intersegmental vessels.

Apelin stimulates angiogenesis in the chicken chorioallantoic membrane (CAM) assay system

The chick CAM is an established in vivo model system for assessment of angiogenic activity (Brooks et al., 1994; Ribatti et al., 1995; 1997). We have carried out a series of experiments in which filters soaked in control medium (PBS) or medium supplemented with apelin or VEGF were placed in contact with the CAM at approximately 10 days after fertilization. After 3 days of incubation, the degree of angiogenic stimulation was assayed by counting the number of vascular branch points (Wilting et al., 1991). Representative examples of control and growth-factor-treated CAMs are shown in Figs. 5A–C. Quantitation of the results of 3 separate experiments, each including at least 7 individual CAMs, is presented in Fig. 5D. These data show that apelin (10 ng/ml) stimulates angiogenic branching approximately 2.5-fold relative to controls. The stimulation of angiogenic growth is approximately equivalent to that induced by treatment with VEGF (50 ng/ml).

Fig. 4. Antisense morpholino inhibition of apelin/APJ signaling in the frog embryo leads to vascular patterning defects. (A) Control experiments were carried out as previously described (Small et al., 2005) to demonstrate effective inhibition of translation. Briefly, fusion transcripts contained 5′ UTR sequences of the target mRNAs upstream of the EGFP coding region. Fluorescence was visualized under UV illumination. (B–G) Embryos were injected with antisense morpholino into one cell of the two-cell embryo. The uninjected side of the embryo serves as a stage-matched control. In all cases, structure of vascular tissue was assayed by in situ hybridization using probe for erg transcripts. (B, C) Control and injected side respectively of an embryo treated with apelin antisense morpholino (apelin MO1). Note reduction in intersegmental vessels on the MO-treated side (dashed arrows), while the majority of vascular structures appear to be unaffected. (D, E) Control and treated side respectively of an embryo injected with a second apelin antisense MO (apelin MO2), again showing disruption of intersegmental vessels in the injected side. (F, G) Control and treated side respectively of an embryo injected with APJ antisense MO. Once again, growth of intersegmental vessels is disrupted on the injected side (dashed arrows). (H) Coinjection of APJ mRNA partially rescues intersomitic defects generated using APJ MO1. Rescue with both 200 and 400 pg of APJ mRNA is statistically significant using the Chi-squared test.


A
apelin UTR-GFP
APJ UTR-GFP
control
apelin MO1 (15ng)
apelin MO2 (7.5ng)
control
APJ MO (7.5ng)
Bright field
UV
Control side
MO treated side
B
C
apelin MO1
erg probe
D
E
apelin MO2
F
G
apj MO1
H
Intersomitic defects (%)
100
80
60
40
20
0
67%
n=24
41%
n=37
32%
n=25
APJ MO
APJ MO + 200 pg mRNA
APJ MO + 400 pg mRNA

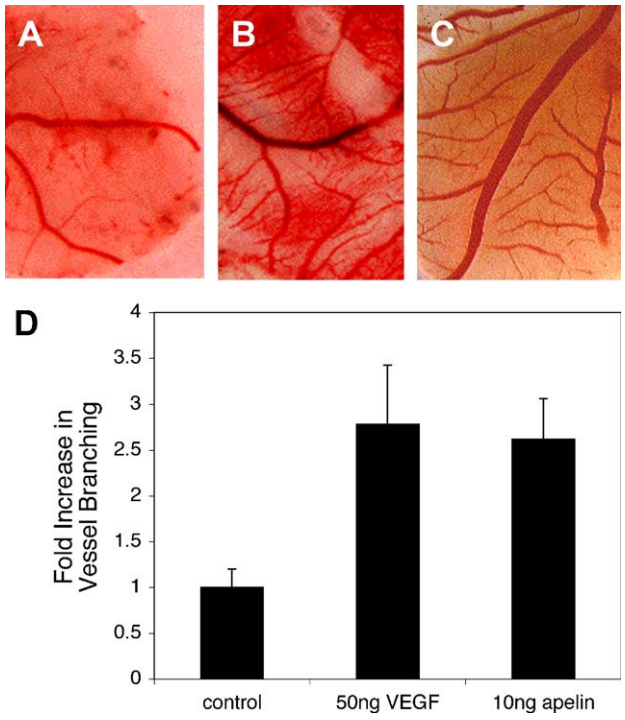


Fig. 5. Apelin is an angiogenic factor in the chicken chorioallantoic membrane (CAM) assay. (A) Photograph of CAM treated with buffer only. (B) Photograph of representative CAM treated with VEGF and (C) representative CAM treated with apelin. (D) Angiogenic activity was quantitated by counting the number of vascular branch points resulting after each treatment. The bar indicates the average of 3 different experiments, each counting at least 7 treated CAMs. The standard error is indicated. Both VEGF and apelin stimulate approximately 2.5-fold increase in angiogenic growth in the CAM assay.

Apelin is a mitogen for endothelial cells in culture

The results of in vivo studies described above suggest that apelin stimulates proliferation and migration of vascular endothelial cells, however, in the complex environment of the embryo, it is very difficult to determine whether the observed effects are direct or indirect. To specifically address the effects of apelin signaling on endothelial cells, we have carried out a number of studies using cells in culture. Primary human umbilical vascular endothelial cells (HUVECs) are commonly used for growth factor studies, but, in agreement with a previous report (Kasai et al., 2004), we found that these cells do not express detectable amounts of APJ. Examination of a number of endothelial cells lines established that the mouse brain microvasculature-derived endothelial cell line bEnd.3 expressed significant amounts of the apelin receptor, APJ, and the VEGF receptor, VEGFR-2 (data not shown), and therefore we have used this cell line to investigate the angiogenic properties of apelin/APJ signaling. Dose curves for proliferation of bEnd.3 cells stimulated with VEGF(165) and apelin(13) are presented in Fig. 6A. VEGF treatment at 10 ng/ml increases bEnd.3 cell proliferation about 2-fold over serum-free control levels, and this factor does not increase significantly when the concentration of VEGF is raised to 100 ng/ml. Apelin treatment at 0.1 ng/ml produces a significant increase in proliferation rate over serum-free controls (1.6-fold), and the rate of BrdU incorporation reaches approximately 2-fold over control at 1 ng/ml. Increasing apelin concentrations to 100 ng/ml does not produce any further increase in cell proliferation. The monomeric apelin (13) peptide has an MW of approximately 1.55 kDa, whereas dimeric VEGF(165) has an MW of about 38 kDa. Based on this 25-fold difference in mass, the dose curve in Fig. 6A suggests that, in molar terms, apelin is almost as effective as VEGF in stimulating endothelial cell proliferation. As a result of these in vitro studies, it appears that the amounts of apelin used in the embryonic bead implantation experiments (Fig. 3) may have been much greater than necessary to elicit an angiogenic response.

Apelin-induced cell proliferation is independent of VEGF and FGF receptor signaling

Using cell proliferation as an assay, we have further investigated whether apelin is a direct stimulator of angiogenesis or whether its effects may be mediated through upregulation of other potent angiogenic growth factors, specifically VEGF and FGF. To examine this possibility, we have inhibited activity of the VEGF and FGF receptors using the highly specific tyrosine kinase inhibitors SU1498 and SU5402 respectively (Shen et al., 1999; Mohammadi et al., 1997). As shown in Fig. 6B, addition of VEGF(165) at 50 ng/ml stimulates bEnd.3 cell proliferation approximately 2.5-fold over control levels, but this stimulation is reduced to almost background levels when VEGF signaling is inhibited by 25 μM SU1498. In contrast, cell proliferation induced by apelin at 10 ng/ml is not significantly inhibited by addition of SU1498. This result suggests that apelin-stimulated cell proliferation is not dependent on VEGF receptor function. Similar experiments show that addition of bFGF at 50 ng/ml increases bEnd.3 proliferation approximately 2-fold over background levels and that this increase can be effectively eliminated by co-treatment with the FGF receptor inhibitor SU5402 at 20 μM. Once again, apelin stimulation of cell proliferation is not significantly reduced in the presence of SU5402. Taken together, these results indicate that the mitogenic activity of apelin is not mediated via the VEGF or FGF receptor pathways.

Apelin stimulates migration of endothelial cells and acts as an anti-apoptotic agent

Using a standard trans-membrane migration assay in a modified Boyden chamber, we have investigated the ability of apelin to stimulate migration of endothelial cells. The results show that apelin at 10 ng/ml stimulates bEnd.3 cell migration approximately 2-fold over serum-free control levels (Fig. 7A). This is approximately the same stimulation of migration as that generated by VEGF (50 ng/ml). We have also investigated the ability of apelin to inhibit programmed cell death (Fig. 7B). Using a standard serum starvation protocol, our results illustrate that supplementation of serum-free medium with apelin is able to inhibit apoptosis (measured by TUNEL assay) to

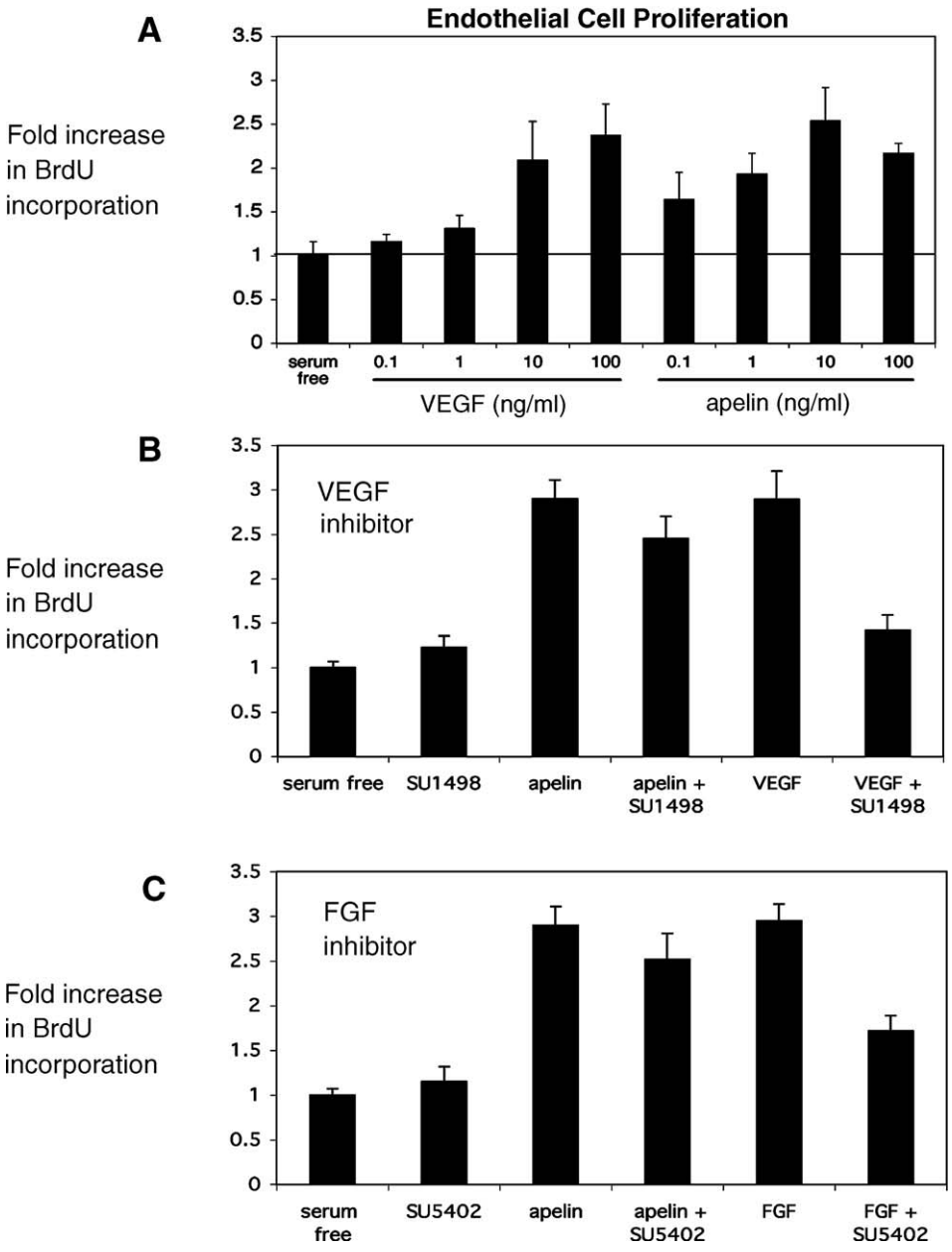


Fig. 6. Apelin is a mitotic factor for endothelial cells in culture and acts independently of VEGF or FGF signaling. All studies were carried out using the mouse bEnd.3 cell line. (A) BrdU incorporation was used to assay proliferative response to different doses of apelin and VEGF(165). (B) SU1498 was used to inhibit VEGF/VEGFR2-mediated proliferation. Note that SU1498 strongly inhibited VEGF-stimulated proliferation but did not significantly affect apelin-stimulated proliferation. Apelin and VEGF were present at 10 ng/ml and 50 ng/ml respectively. (C) SU5402 was used to inhibit signaling through the FGF receptor. SU5402 strongly inhibited FGF-stimulated proliferation of bEnd.3 cells but had no significant effect on apelin-stimulated proliferation. Apelin and bFGF were present at 10 ng/ml and 100 ng/ml respectively. Panels show mean of 3 experiment plus SEM.

approximately 40% of control levels (Fig. 7B). Using the same procedure, VEGF at 50 ng/ml reduces apoptosis to about 30% of control levels.

Apelin expression is induced by hypoxia in primary cardiac myocyte cell culture

It has been noted that transcription of many angiogenic growth factors is induced by low oxygen conditions (reviewed in Wenger, 2002; Pugh and Ratcliffe, 2003). In order to provide a thorough characterization of apelin as an angiogenic factor, we have tested whether apelin transcription is activated in rat cardiac myocytes treated with cobalt chloride (Ho and Bunn, 1996; Zhu and Bunn, 2001). Cobalt ions stabilize the hypoxia inducible factor 1α (HIF-1α) transcription factor, thereby activating the same transcription programs that are stimulated by hypoxia (Ho and Bunn, 1996). Cardiac myocytes were used for these experiments because they express significant levels of apelin (Szokodi et al., 2002 and data not shown) and because they have previously been demonstrated to activate angiogenic gene expression in response to cobalt chloride treatment (Weil et al., 2003; Chun et al., 2003). As shown in Fig. 7C, after 4 h of

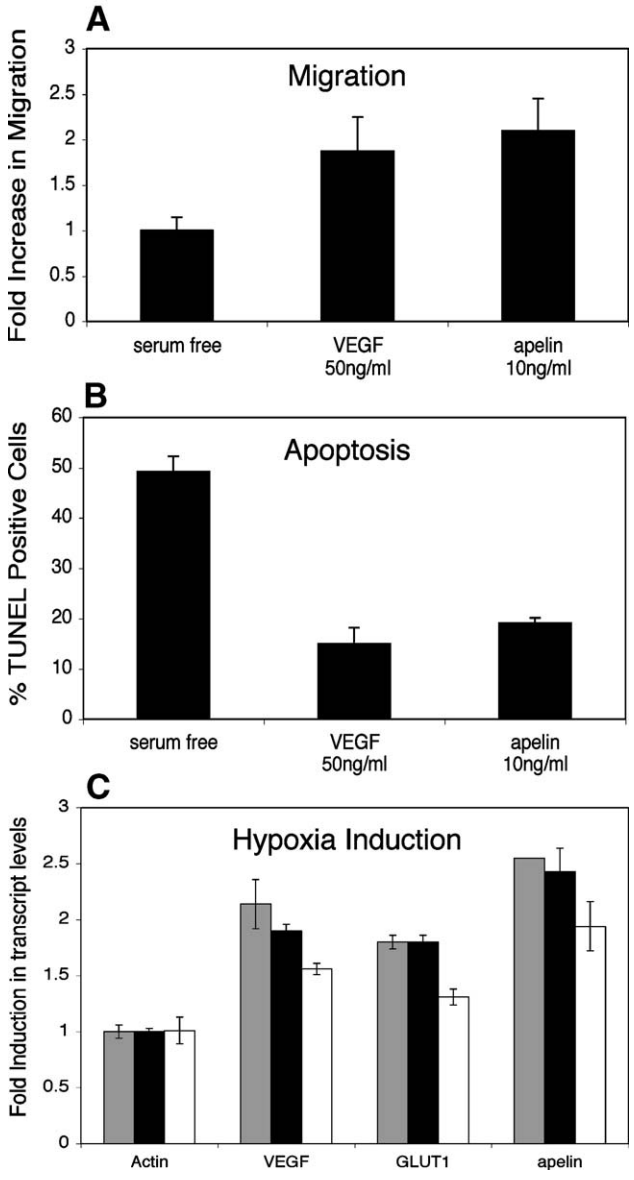


Fig. 7. Apelin stimulates cell migration and is anti-apoptotic for endothelial cells. Studies were carried out using mouse bEnd.3 cells. (A) A modified Transwell assay was used to measure growth-factor-mediated migration. Both VEGF and apelin-stimulated migration approximately 2-fold over control levels. The mean of 3 experiments plus SEM is shown. (B) Apoptosis was measured using TUNEL assay after 3 days serum starvation. Both VEGF and apelin inhibit apoptosis. The mean of 3 experiments plus SEM is shown. (C) Apelin transcription is induced by treatment mimicking hypoxia. Real-time PCR analysis of transcript levels following treatment of primary rat cardiomyocytes with $CoCl_2$ solution. Cytoskeletal β-actin was used as a normalization control, and VEGF and GLUT1 are positive controls for hypoxia responsive genes. Results of three different experiments, each in triplicate, plus SEM are shown.

incubation in the presence of 125 μM $CoCl_2$, apelin transcript levels increased approximately twofold relative to cytoskeletal β-actin transcripts. Expression of β-actin has previously been shown to be unresponsive to hypoxia (Chun et al., 2003; Vengellur and LaPres, 2004). For positive controls, we assayed VEGF and GLUT1 transcripts, both of which are known to be upregulated by hypoxia (Minchenko et al., 1994; Behrooz and Ismail-Beigi, 1997). Under our experimental conditions, levels of both transcripts were also increased about twofold (Fig. 7C). Based on these experiments, we conclude that transcription of apelin is increased under conditions of hypoxia and is regulated by HIF-1α. This model is supported by the observation that multiple HIF-1α binding sites are present in the promoter and first intron of the apelin genes of mouse, chicken, zebrafish and frog (data not shown). Although these experiments indicate possible activation of apelin in hypoxia, we do not wish to imply that hypoxia is involved in embryonic expression of apelin in the developing vasculature.

Discussion

Developmental expression of apelin/APJ

The G-protein-coupled receptor APJ is expressed at high levels in endothelial cells of the developing vasculature of frog and mouse embryos (Devic et al., 1996; 1999; Cleaver et al., 1997; Fig. 2). Analysis of apelin ligand expression (Fig. 2) shows that apelin and APJ appear to be co-expressed in a subset of endothelial cells in the *Xenopus* embryo, although we cannot strictly exclude the possibility that apelin expression occurs in cells adjacent to vascular structures rather than endothelial cells themselves. This apparent co-expression occurs in the developing posterior cardinal veins, the vessels surrounding the eye and the branching intersegmental vessels. Apelin expression in at least some of these domains is transient. For example, apelin is expressed in the blood vessels dorsal to the eye at stage 32, but approximately 10 h later, at stage 35, apelin transcripts in this region are undetectable. Apelin expression in the intersegmental vessels is also strong but transient. The co-expression of apelin and APJ in endothelial cells raises the possibility of autocrine signaling during development of a subset of embryonic blood vessels. It should be emphasized, however, that there are also a significant number of examples where the apelin and APJ expression domains are not coincident. For example, although APJ is strongly expressed in the individual endothelial precursor cells in the ventral plexus region and the aortic arches (Fig. 2A), apelin expression is not observed at significant levels in these regions. Similarly, although not domains of vascular development, the ventral neural tube expresses high levels of apelin (Figs. 2F, G), but no APJ expressing cells are in the immediate vicinity. The lack of overlap of apelin and APJ expression domains in some regions of the embryo raises the possibility that additional ligands and/or receptors may participate in the apelin/APJ signaling pathway. Alternatively, given the ability of apelin to act as a chemotactic agent for endothelial cells (Fig. 7A), the localized sources of apelin in the embryo may help to regulate angiogenic outgrowth into avascular tissues during subsequent development.

Apelin/APJ signaling regulates Xenopus vascular development

We have tested for a developmental function for apelin/APJ signaling using a morpholino knockdown strategy. These

experiments consistently yielded defects in development of the intersegmental vessels. Identical phenotypes were obtained when transcripts encoding either apelin (two MOs) or the APJ receptor were targeted, suggesting that the observed defects were specific and unlikely to be due to generalized perturbations of normal embryonic development. Somewhat surprisingly, other regions of the developing vasculature appeared normal, implying that apelin/APJ signaling is primarily involved in development of the intersegmental vessels and not for other prominent vessels, such as those surrounding the eyes, which also co-express significant levels of apelin and APJ. Recently, APJ function in the mouse has been ablated by homologous recombination (Ishida et al., 2004). The mice are viable and fertile and show no obvious defects in vascular development or function, although the animals do exhibit increased vascular contractility in response to blood pressure regulators. At least two explanations may account for the different results observed in the frog and mouse loss of function studies. First, the mouse may possess redundant signaling activities that are not present in the frog embryo. These putative signaling molecules might be an alternative to the APJ receptor that can bind the apelin ligand or may represent an unrelated signaling pathway that shares angiogenic activity. Indeed, the possibility of additional signaling or receptor molecules is consistent with the observed non-congruence of apelin and APJ expression domains already noted in the frog embryo. Second, the inhibition of intersegmental vessel growth observed in the frog may be a transient effect that is corrected during later development. In this interpretation, the mouse embryos may also have exhibited early vascular defects, but these were not evident at postnatal stages when the animals were studied. Since MOs cease to be effective fairly early in frog embryonic development (Heasman et al., 2000), it is not possible to determine long-term knockdown effects using this model system.

Apelin acts independently of VEGF signaling

We have used both frog embryos and chick CAM assays to demonstrate that apelin possesses angiogenic activity in vivo. In these experiments, apelin/APJ signaling may directly activate endothelial cell migration and proliferation or it may be acting through upregulation of another angiogenic factor. For example, both thrombin and angiotensin II, signaling through the receptors PAR-1 and AT-1R respectively, demonstrate angiogenic properties in vivo and in vitro, but at least a proportion of these activities appear to be mediated through upregulation of the VEGF signaling pathway (Richard et al., 2000; Williams et al., 1995). Similarly, the growth factor TWEAK, acting through the Fn14 receptor, has been shown to potentiate the mitogenic activity of FGF-2 and VEGF for endothelial cells (Donohue et al., 2003). We have specifically addressed the question whether apelin exerts its effects through upregulation of VEGF expression in the frog embryo. In situ hybridization detection of VEGF transcripts shows no detectable expression of VEGF in the vicinity of the apelin bead (Fig. 3H), suggesting that apelin does not act via transcriptional upregulation of VEGF activity. This general result has been confirmed under more controlled conditions in experiments using cultured cells. When VEGF signaling is suppressed using the VEGFR2 kinase inhibitor SU1498, apelin-stimulated proliferation of endothelial cells is not significantly inhibited. Similarly, inhibition of FGF signaling using the receptor tyrosine kinase inhibitor SU5402 does not result in inhibition of cell division regulated by apelin. These studies do not preclude the possibility that apelin activates expression of other factors capable of stimulating endothelial cell division, but the results suggest that such effects are not regulated via simple upregulation of expression of VEGF-A or FGF family growth factors.

G protein signaling and vascular function

In whole animal studies, apelin has been shown to regulate a range of physiological activities including regulation of fluid and food uptake, modulation of vascular tone and regulation of heartbeat. Apelin therefore joins an intriguing group of GPCR ligands that exhibit angiogenic properties and also a range of other, apparently unrelated physiological activities. For example, prokineticin-1 (also called EG-VEGF) is an 86 aa protein that binds to the PK1 receptor and stimulates smooth muscle contraction and is also a potent angiogenic agent (Le Couter et al., 2001; Li et al., 2001). Angiotensin II is an 8 amino acid peptide that possesses angiogenic properties and also regulates physiological pathways that modulate blood pressure, fluid and sodium retention and cardiac function (Touyz, 2003). Similarly, adrenomedullin exhibits potent vasodilator and diuretic activity (Brain and Grant, 2004) in addition to angiogenic properties (Oehler et al., 2002). It seems reasonable to assume that the angiogenesis-stimulating properties of these factors are not directly related to their vasoregulatory roles under normal physiological conditions. Our results show that apelin is a mitogen for endothelial cells in vivo (Figs. 3, 5) and in vitro (Fig. 6) and that apelin possesses chemotactic activity for endothelial cells (Fig. 7A) and is anti-apoptotic for endothelial cells in culture (Fig. 7B). These data are in broad agreement with a previous study using mouse retinal endothelial cells (Kasai et al., 2004). Furthermore, apelin expression is increased in cells cultured under conditions mimicking hypoxia (Fig. 7C). This latter result suggests that apelin expression may be upregulated during the hypoxic conditions associated with early tumor growth (Folkman, 1995) and may have the ability to stimulate the growth or stabilization of tumor blood vessels. Overall, our studies suggest that apelin/APJ signaling is required for normal development of the vascular system in the frog embryo. Furthermore, in addition to its known functions in regulating heart and vascular physiology, apelin has the potential to modulate normal and pathological blood vessel growth in the mature organism.

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