Human Angiogenin Is Rapidly Translocated to the Nucleus of Human Umbilical Vein Endothelial Cells and Binds to DNA

Guo-fu Hu, Chi-jie Xu, and James F. Riordan*

Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, Boston, Massachusetts 02115

Abstract   Human angiogenin is translocated to the nucleus of human umbilical vein endothelial cells in a time-dependent manner. Exogenous angiogenin appears in the nucleus in 2 min, reaches saturation in 15 min when 85% of the internalized angiogenin is in the nuclei, and remains associated with the nucleus for at least 4 h. Endothelial cells cultured at low density have a much higher capacity to translocate angiogenin to the nucleus than do those cultured at high density. This observation is consistent with previous findings that both the ability of endothelial cells to proliferate in response to angiogenin and the expression of an angiogenin receptor on the cell surface depend on cell density. Nuclear ^{125}I-angiogenin is not degraded and is neither spontaneously dissociated nor replaced by unlabeled angiogenin. It is, however, released by deoxyribonuclease I, but not by ribonuclease A, suggesting that angiogenin binds to DNA in the nucleus. These results suggest that in addition to acting as a ribonuclease, angiogenin may play a role in regulating gene expression by direct binding to DNA. J. Cell. Biochem. 76:452–462, 2000.   © 2000 Wiley-Liss, Inc.

Key words: human angiogenin; angiogenesis; human endothelial cells; nuclear translocation; cell density

Angiogenin belongs both to a class of direct inducers of angiogenesis [Bussolino et al., 1997] and to the ribonuclease superfamily [Riordan, 1997]. Its angiogenic activity relies on its interaction with endothelial cells, as well as on its activity as a ribonucleolytic enzyme [Shapiro et al., 1986]. Angiogenin is pleiotropic to endothelial cells. It binds to a 170-kDa receptor [Hu, 1997] and to a 42-kDa binding protein [Hu et al., 1993] on the endothelial cell surface, induces cell proliferation [Hu, 1997], activates cell-associated proteases [Hu and Riordan, 1993], and stimulates cell migration and invasion [Hu et al., 1994]. It also mediates cell adhesion [Soncin, 1992] and promotes tube formation by cultured endothelial cells [Jimi et al., 1995]. All these individual cellular events are necessary components of the process of angiogenesis. Intervention at any of these steps would provide a means to control the activity of angiogenin. Indeed, the cytosolic ribonuclease inhibitor, which abolishes the ribonucleolytic activity of angiogenin [Shapiro and Vallee, 1987], and the cell surface binding protein of angiogenin, as well as anti-angiogenin monoclonal antibodies, are all potent antagonists of its angiogenic activity [Hu et al., 1993].

A striking feature of angiogenin is that it normally circulates in human plasma with a concentration range of 250–360 ng/ml [Bläser et al., 1993; Shimoyama et al., 1996]. In mouse plasma, it has a half-life of ~5 min [Vasandani et al., 1996]. The plasma concentration rises in a number of pathological conditions and in patients with a variety of tumors [Li et al., 1994; Shimoyama et al., 1996; Barton et al., 1997; Chopra et al., 1997, 1998]. The presence of angiogenin in plasma suggests, on the one hand,

that it plays an important role under normal physiological circumstances but, on the other hand, that attempts to neutralize it to control unwanted angiogenesis might be both complicated and inefficient. The identification of nuclear translocation of angiogenin in endothelial cells and the demonstration of its necessity for angiogenesis [Moroianu and Riordan, 1994a,b] provide several additional opportunities for controlling the activity of angiogenin. Inhibitors that target the process of nuclear translocation would not encounter the potential problems caused by circulating angiogenin in plasma. Since translocation of angiogenin from the exterior of the cell to the nucleus is a multistep process that involves internalization, transport across the cytoplasm, and entrance into the nucleus, blocking any one of these steps would result in inhibition of nuclear translocation as well as cell proliferation and angiogenesis. Neomycin, an aminoglycoside antibiotic, has been shown to block nuclear translocation of angiogenin in endothelial cells, thereby abolishing its angiogenic activity [Hu, 1998].

Internalization of angiogenin by endothelial cells is energy- and temperature-dependent, suggesting receptor-mediated endocytosis [Moroianu and Riordan, 1994a]. Transport of internalized angiogenin across the cytoplasm seems to involve an endosomal route that is independent of the microtubule network and lysosomal processing [Li et al., 1997]. A nuclear localization sequence (NLS) has been identified on angiogenin and shown to be necessary for nuclear translocation and angiogenesis [Moroianu and Riordan, 1994c]. Angiogenin variants with an altered NLS have full ribonucleolytic activity and capacity to bind to the endothelial cell surface, but they do not undergo nuclear translocation and are not angiogenic [Moroianu and Riordan, 1994a]. Requirement of an NLS suggested that translocation of angiogenin to the nucleus occurs by a classic nuclear pore route and is mediated by an NLS-binding protein. Despite the preliminary knowledge outlined above, very little is known about the details of the process by which angiogenin is endocytosed, transported to the nuclear membrane, released into the nucleus, localized to the nucleolus, and bound to its ultimate target molecules.

It is also unknown what angiogenin does in the nucleus. It seems that angiogenin exerts its characteristic ribonucleolytic activity upon its arrival in the nucleus. Recently, a DNA aptamer for angiogenin was obtained by a screening procedure known as "systematic evolution of ligands by exponential enrichment" and was shown to inhibit both the ribonucleolytic and angiogenic activities. Interestingly, this aptamer is co-translocated to the nucleus of endothelial cells with angiogenin, suggesting that it exercises its inhibitory activity in the cell nucleus [Nobile et al., 1998].

Understanding the mechanism of nuclear translocation of angiogenin in endothelial cells might lead to the design of more efficient angiogenesis inhibitors. We have begun to address these issues by establishing a quantitative assay for nuclear translocation of angiogenin in human endothelial cells by means of ligand iodination and subcellular fractionation. This has allowed us to characterize the kinetics of nuclear translocation, subcellular distribution, and dissociation of nuclear angiogenin.

MATERIALS AND METHODS
Materials

Angiogenin was prepared as a human recombinant protein [Shapiro et al., 1988]; acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) were from Promega (Madison, WI); human endothelial serum-free medium (HE-SFM) was from G-BRL-Life Technologies; fetal bovine serum (FBS) was from Hyclone (Logan UT); excellulose GF-5 desalting columns and Iodo-Beads iodination reagents were from Pierce (Rockford, IL); $Na^{125}I$ (17.4 Ci/mg) and methyl-^{3}H- thymidine (6.7 Ci/mmol) were from Dupont/NEN; heparin (MW 6,000), ribonuclease A (RNase A), deoxyribonuclease I (DNase I) and micrococcal nuclease were from Sigma Chemical Co. (St. Louis, MO); complete protease inhibitor cocktail was from Boehringer-Mannheim (Indianapolis, IN).

Cell Culture

Human umbilical vein endothelial (HUVE) and human umbilical artery endothelial (HUAE) cells were purchased from Cell Systems as primary cultures isolated from human umbilical veins and arteries, respectively. The cells were cultured in HE-SFM supplemented with 10% FBS and 10 ng/ml bFGF at 37°C under 5% humidified CO_2 and were split 1:4 for subculture. Cells between passages 5 and 10 inclusive were used for all experiments. Cell

numbers were determined with a Coulter counter, and cell viability was measured by the trypan blue dye exclusion assay.

Iodination of Angiogenin

^{125}I-angiogenin was prepared with the use of Iodo-beads. One Iodo-bead was added to 175 µl of 0.114 M phosphate buffer, pH 6.5, containing 0.5 mCi Na^{125}I and incubated at room temperature for 5 min. Angiogenin, 50 µg in 25 µl H$_2$O was added, and the mixture was incubated at room temperature for another 15 min. The reaction was terminated by removing the Iodo-bead, and iodinated angiogenin was separated from free iodine by a GF-5 desalting column equilibrated in 0.1 M phosphate buffer, pH 6.5. Fractions of 0.5 ml were collected, and the radioactivity in each fraction was determined with a gamma counter. Angiogenin iodinated by this procedure has a specific activity of 1–2 × 10^6 cpm/µg and contains 5–6% dimeric form, as demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. The areas corresponding to the monomeric and dimeric forms of ^{125}I-angiogenin were incised and radioactivities determined by a gamma counter.

Cell Proliferation

Cells were seeded in attachment factor (from Cell Systems Corp.)-coated 35-mm dishes in HE-SFM at 5 × 10^3 cells/cm^2. Angiogenin samples (1 µg/ml) were added immediately after the cells were seeded. The cells were incubated at 37°C in humidified air containing 5% CO$_2$ for 48 h. At the end of this time, the medium was aspirated and the cells were washed once with 1 ml phosphate-buffered saline (PBS) and detached with 0.25 ml of trypsin-versene (0.05%) solution. Cell numbers were determined with a Coulter counter.

Nuclear Translocation

Cells were seeded at 5 × 10^3/cm^2 in 35-mm dishes and cultured in HE-SFM supplemented with 20 ng/ml bFGF at 37°C under 5% humidified CO$_2$ for 24 h. The cells were washed three times with prewarmed (37°C) HE-SFM and incubated with angiogenin (50 ng to 1 µg/ml) at 37°C for 30 min. Immunofluorescent staining of nuclear angiogenin was performed as described previously [Moroianu and Riordan, 1994a].

Briefly, fixed cells were washed 3 times with phosphate-buffered saline containing 30 mg/ml bovine serum albumin (BSA) for 10 min at 37°C and incubated with 50 µg/ml of anti-angiogenin monoclonal antibody in PBS containing 5 mg/ml BSA at 37°C for 1 h. The cells were then washed 5 times with PBS + 5 mg/ml BSA at 37°C and incubated with fluorescein isothiocyanate-labeled goat F(ab′)$_2$ anti-mouse IgG at 1:100 dilution in PBS + 5 mg/ml BSA for 1 h at 37°C. After incubation, the cells were washed 5 times with PBS + 5 mg/ml BSA, once with PBS and then mounted in 50% glycerol. Fluorescence was observed with a Nikon Labphot fluorescent microscope. Photographs were taken with a Nikon AFX-II autoexposure system. When ^{125}I-angiogenin was used, the cells were washed 3 times with PBS after incubation. Membrane-bound ^{125}I-angiogenin was dissociated by washing with PBS, pH 3.0; cytosol-accumulated ^{125}I-angiogenin was collected by solubilizing the cells with 0.5% Triton X-100 in PBS, pH 7.5; distilled H$_2$O was added to the remaining material (nuclei + extracellular matrix) and the swollen nuclei were separated from extracellular matrix by gentle pipetting. Nuclei isolated by this method are essentially free of extracellular matrix [Savion et al., 1981]. However, since ^{125}I-angiogenin is known to bind extracellular matrix components [Badet et al., 1989], an alternative method was also used to obtain the nuclear fraction to ensure that the nuclei isolated were not contaminated by ^{125}I-angiogenin dissociated from extracellular matrix. Cells were detached by scraping with a rubber cell scraper and centrifuged at 800g for 5 min. The cell pellets were washed once with cold PBS and lysed with 0.5% Triton X-100 in PBS containing ×1 complete protease inhibitor cocktail at 4°C for 30 min. The cell solubilizates were centrifuged at 1,200g to pellet the nuclear fraction that was washed twice with PBS. Radioactivity in each fraction was determined with a gamma counter. The integrity and quality of the nuclei were evaluated by light microscopy. Similar results were obtained with the two nuclei isolation procedures.

RESULTS

Angiogenin Is Translocated to the Nucleus of HUVE Cells

HUVE cells were chosen for this study because they are relatively easy to culture and

Fig. 1. Immunofluorescent detection of nuclear angiogenin in human umbilical vein endothelial (HUVE) cells. Cells were incubated with 1 μg/ml of human angiogenin for 30 min at 37°C and immunostained with anti-angiogenin monoclonal antibody 26-2F and fluorescein isothiocyanate-labeled goat F(ab′)$_2$ anti-mouse IgG. ×100.
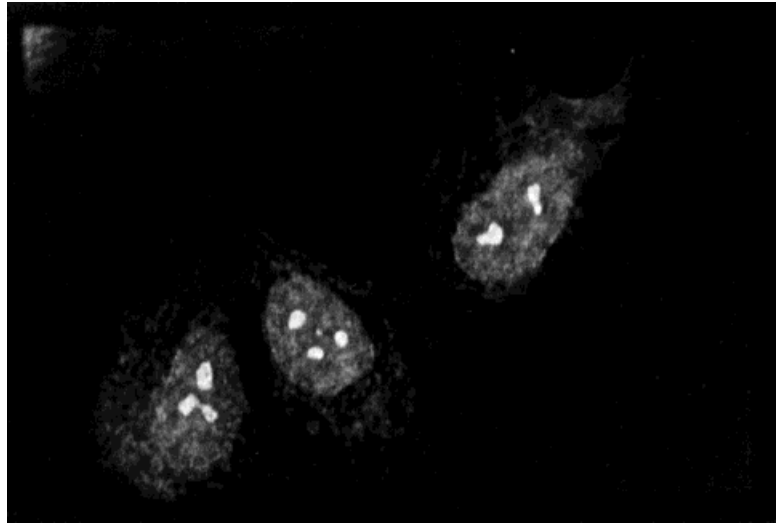
TABLE I. Mitogenic Activity of Iodinated Angiogenin*

	Control	Angiogenin	^{125}I-Angiogenin	^{127}I-Angiogenin
Cell number	45,900 ± 1,700	57,600 ± 1,500	61,300 ± 2,800	63,200 ± 3,100
% of control	100	126	134	138

*Human umbilical vein endothelial (HUVE) cells, seeded at 50,000 cells per dish on attachment factor-coated 35-mm dishes in HE-SFM and stimulated with various forms of angiogenin (1 μg/ml) at 37°C for 48 h [Hu et al., 1997]. Cells were released by trypsinization and cell numbers were determined with a Coulter counter. Data are the mean of quadruplicates from one of the two repeated experiments.

have been shown to be most responsive upon stimulation of exogenous human angiogenin [Hu et al., 1997]. Nuclear translocation of human angiogenin in HUVE cells was first visualized by indirect immunofluorescence. Immunofluorescent staining with an anti-angiogenin monoclonal antibody showed that exogenous angiogenin accumulates in the nucleus of proliferating HUVE cells after 30-min incubation at 37°C (Fig. 1). No nuclear angiogenin was detected in controls that were not exposed to exogenous angiogenin or were stained with a nonimmune monoclonal antibody (data not shown).

Nuclear Translocation of Angiogenin in HUVE Cells Is Rapid

A more quantitative assay would be necessary to characterize the translocation kinetics, subcellular distribution of internalized angiogenin, and dissociation of nuclear angiogenin. These issues are pertinent to the mechanism of nuclear translocation and were addressed by means of ligand iodination and subcellular fractionation. We first established that iodinated angiogenin behaves in the same manner as unlabeled angiogenin in cell proliferation assays. Angiogenin-induced cell proliferation involves binding of angiogenin to its receptor, propagation of proper intracellular signals, as well as nuclear translocation of angiogenin and is, therefore, an appropriate measure of the biological function of angiogenin. Table I shows that both ^{125}I- and ^{127}I-angiogenin actively induce HUVE cell proliferation. Cell number increases induced by 1 μg/ml of ^{125}I- and ^{127}I-angiogenin are 34% and 38% over the control, respectively, not significantly different from the 26% increase induced by the same concentration of unlabeled angiogenin.

Having demonstrated that endothelial cells do not distinguish between iodinated and unlabeled angiogenin, we determined the time course of nuclear translocation of angiogenin in HUVE cells by the use of ^{125}I-angiogenin. As shown in Figure 2, exogenous angiogenin rapidly translocates to the nucleus of HUVE cells in a time-dependent manner. Nuclear ^{125}I-angiogenin was detected after 2-min incubation, increased progressively, reached a maximum at 15 min, and remained unchanged for at least 4 h.
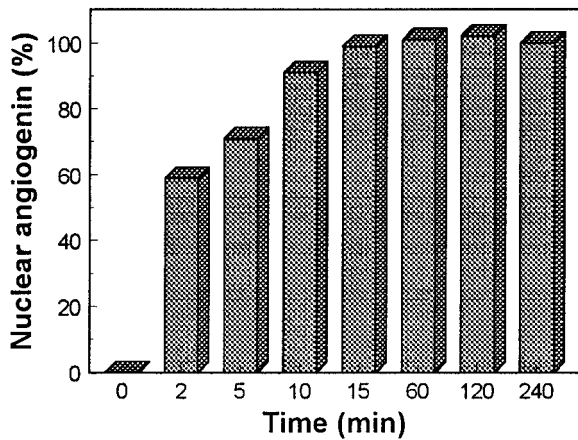
Fig. 2. Nuclear translocation of angiogenin is rapid. Human umbilical artery endothelial (HUAE) cells, 100,000 cells per 35-mm dish, were cultured in CS-5.0 for 20 h, washed once with human endothelical serum-free medium (HE-SFM) and continued in culture in HE-SFM for another 20 h. ^{125}I-angiogenin, 200 ng/ml, was added to the cells and incubated for different periods of time. Nuclear fractions were isolated and radioactivity was determined. The radioactivity at 60 min was considered as 100%. Data shown are means of duplicates.

TABLE II. Nuclear Translocation of ^{125}I-Angiogenin Is Cell Density Dependent*

Density (cells/cm^2)	Cell No.	Nuclear ^{125}I-angiogenin (cpm)	Nuclear ^{125}I-angiogenin per 10^3 cells (cpm)
5,000	50,000	3,200 ± 200	64
10,000	100,000	2,530 ± 300	25
15,000	150,000	1,900 ± 200	13
20,000	200,000	1,300 ± 10	6.5

*Human umbilical vein endothelial (HUVE) cells, at different densities, were incubated with 1 µg/ml ^{125}I-angiogenin at 37°C for 30 min. Nuclear fractions were isolated, and ^{125}I-angiogenin was determined with a gamma counter. Data shown are means and ranges of duplicates of each sample in a representative experiment.

Nuclear Translocation of ^{125}I-Angiogenin Depends on Cell Density

Endothelial cells express an angiogenin receptor and respond to exogenous angiogenin only when they are in sparse culture [Hu et al., 1997]. We investigated the effect of cell density on nuclear translocation by measuring the amount of nuclear angiogenin in the cells cultured under various densities. As shown in Table II, the ability of HUVE cells to translocate angiogenin to the nucleus decreases as the cell density increases. Cells cultured at a density of 20,000 cells per cm^2 have only 10% of the

TABLE III. Effect of Culture Conditions on Nuclear Translocation of Angiogenin*

Culture condition	Nuclear ^{125}I-angiogenin (cpm)
CS-5.0^a	110 ± 20
HE-SFM + 10% FBS + 20 ng/ml bFGF	590 ± 10
HE-SFM + 20 ng/ml bFGF	3,000 ± 400

HUVE, human umbilical vein endothelial; HE-SFM, human endothelial serum-free medium; FBS, fetal bovine serum; bFGF, basic fibroblast growth factor; aFGF, acidic fibroblast growth factor.
*HUVE cells, seeded at 50,000 per 35-mm dish, were cultured under the conditions indicated, at 37°C 24 h, washed with prewarmed HE-SFM (37°C) three times and incubated with 1 µg/ml ^{125}I-angiogenin for 30 min at 37°C in HE-SFM. Nuclear fractions were isolated, and radioactivities were determined with a gamma counter. Data shown are means and ranges of duplicates of each sample in a representative experiment.
^{a}Complete medium for human endothelial cells from Cell Systems containing 10% FBS, 50 ng/ml aFGF, and 50 µg/ml heparin.

radioactivity of those cultured at 5,000 cells per cm^2. These results confirm the previous observations that nuclear translocation of angiogenin involves binding to its receptor on the cell surface and that nuclear translocation is required for angiogenin to induce cell proliferation. We also found that nuclear translocation of angiogenin is largely diminished when the cells were cultured in the presence of 10% FBS (Table III). FBS has been shown to inhibit angiogenin-induced ^{3}H-thymidine incorporation and proliferation of HUVE cells [Hu et al., 1997].

Subcellular Distribution of Angiogenin in Endothelial Cells

Table IV shows that after a 30-min incubation, up to 85% of the cell-associated ^{125}I-angiogenin is found in the nuclear fraction both in HUVE and in HUAE cells. Little angiogenin is in the cytosolic fraction, suggesting that angiogenin is not released into the cytosol after internalization. The transport machinery is designed such that angiogenin is not trapped in the cytosol, where a tight binding protein for angiogenin—ribonuclease inhibitor—exists. These data are in agreement with our previous results, which showed that angiogenin is transported from the cell surface to the nucleus in a lysosome- and microtubule-independent manner [Li et al., 1997].

TABLE IV. Subcellular Distribution of Internalized ^{125}I-Angiogenin*

| | ^{125}I-angiogenin (cpm) | | | | | |
Cells	Cell surface	%	Cytosol	%	Nucleus	%
HUVE	350 ± 10	4	900 ± 40	11	6,850 ± 400	85
HUAE	300 ± 20	3	980 ± 20	12	7,100 ± 250	85

HUVE, human umbilical vein endothelial; HUAE, human umbilical artery endothelial.
*^{125}I-angiogenin, 500 ng/ml, was incubated with 50,000 cells cultured in 35-mm dishes at 37°C for 30 min. Cells were washed and fractionated, and radioactivity in each subcellular fraction was determined. Data shown are means and standard deviations of six replicates in a representative experiment.

Nuclear ^{125}I-Angiogenin Is Intact

SDS-PAGE and autoradiography analysis show that the predominant species of nuclear angiogenin has a molecular weight of 14 kDa (Fig. 3). Nuclear ^{125}I-angiogenin appears to be identical among the nuclear fractions prepared after 15 min (lane A), 1 h (lane B), and 4 h (lane C) translocation. The band at 28 kDa represents approximately 20% of the total radioactivity in the nuclear fraction and appears to be a dimeric form of angiogenin that is generated during iodination by an unknown reaction. Iodinated angiogenin has approximately 6% dimeric form. The reason for preferential enrichment of angiogenin dimers in the nucleus is unknown. In any event, there was no sign of degradation, confirming that nuclear translocation is independent of lysosomal processing [Li et al., 1997]. These results also suggest that the function of nuclear angiogenin, no matter what it is, requires its structural integrity.

Effect of Heparin and FGFs on Nuclear Translocation of Angiogenin

Angiogenesis is induced by an array of angiogenic molecules [Hanahan and Folkman, 1996] whose interrelationship remains unclear. FGFs are prototypical angiogenic proteins that also undergo nuclear translocation [Zhan et al., 1992; Bouche et al., 1987] in endothelial cells for angiogenesis [Moroianu and Riordan, 1994b]. However, the nuclear translocation process of angiogenin seems to be independent of that of aFGF and bFGF. As shown in Table V, nuclear translocation of ^{125}I-angiogenin was inhibited by 61% with a 20-fold molar excess of unlabeled angiogenin but unaffected by the same molar
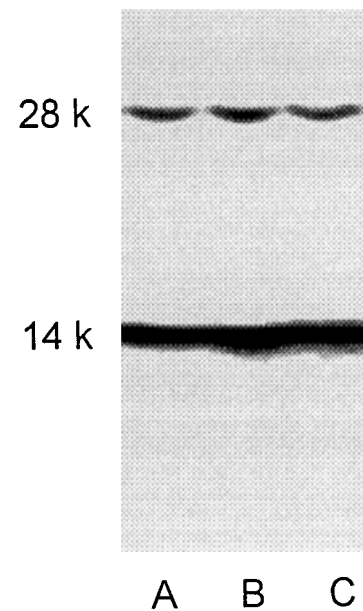

Fig. 3. Nuclear-accumulated angiogenin is intact. Human umbilical vein endothelial (HUVE) cells were cultured at 50,000 cells per 35-mm dish and incubated with 200 ng/ml ^{125}I-angiogenin at 37°C in human endothelial serum-free medium (HE-SFM) for 15 **(A)**, 60 **(B)**, and 240 min **(C)**. Nuclear fractions were isolated and subject to sodium dodecyl sulfate–polyacrylamide gel electrophorisis (SDS-PAGE) and autoradiography.

TABLE V. Effect of Heparin and FGFs on Nuclear Translocation of Angiogenin*

Competitors	Nuclear ^{125}I-angiogenin (cpm)	% of Control	% Inhibition
None	620 ± 20	—	—
Heparin (10 μg/ml)	560 ± 20	90	10
Heparin (100 μg/ml)	390 ± 30	63	37
aFGF (1 μg/ml)	690 ± 40	111	—
bFGF (1 μg/ml)	670 ± 10	108	—
Angiogenin (1 μg/ml)	240 ± 40	39	61

aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; HUVE, human umbilical vein endothelial; HE-SFM, human endothelial serum-free medium.
*HUVE cells, 50,000 cells per 35-mm dish, were cultured in HE-SFM + 20 ng/ml bFGF for 24 h, washed with prewarmed HE-SFM for three times and incubated with 50 ng/ml ^{125}I-angiogenin in the absence or presence of the competitors at 37°C for 30 min. The nuclear fraction was isolated and radioactivity was determined. Data shown were from a representative experiment and are the means and standard deviations of quadruplicates of each sample.

TABLE VI. Release of Nuclear Bound ^{125}I-Angiogenin*

Agents	Released ^{125}I-angiogenin (cpm)			
	1 h	2 h	3 h	24 h
PBS	0	150 ± 10	210 ± 60	$1{,}760 \pm 160$
Angiogenin (10 µg/ml)	0	170 ± 30	260 ± 20	$1{,}440 \pm 220$
RNase A (10 µg/ml)	120 ± 10	140 ± 50	160 ± 30	$1{,}680 \pm 400$
DNase I (10 µg/ml)	870 ± 110	$1{,}350 \pm 50$	$1{,}530 \pm 230$	$4{,}110 \pm 320$

PBS, phosphate-buffered saline; HUVE, human umbilical vein endothelial.
*HUVE cells, 50,000 cells per 35-mm dish, were incubated with 200 ng/ml of ^{125}I-angiogenin for 30 min. The nuclear fraction was isolated and incubated with enzymes for various lengths of time. Radioactivity in the supernatant was determined with a gamma counter. Data shown are the means and ranges of duplicates of each sample. Total counts of ^{125}I-angiogenin in the isolated nuclei from one dish was ~4,000 cpm.
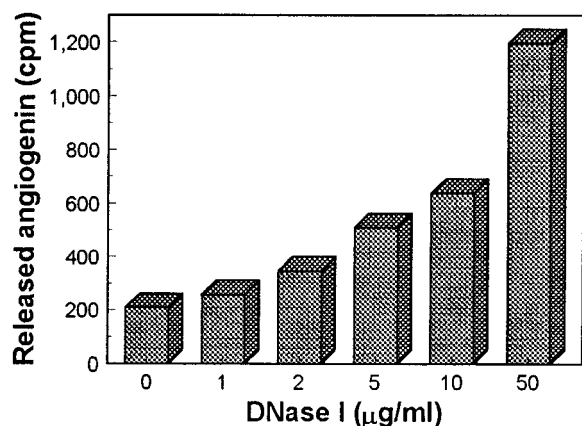

Fig. 4. Release of nuclear bound ^{125}I-angiogenin by DNase I is concentration-dependent. Human umbilical vein endothelial (HUVE) cells, 50,000 cells per 35-mm dish, were incubated with 0.2 µg/ml of ^{125}I-angiogenin at 37°C for 30 min. Nuclear fractions were isolated and incubated with 1, 2, 5, 10, and 50 µg/ml DNase I, respectively, in phosphate-buffered saline (PBS) containing 10 mM $CaCl_2$ and 5 mM $MgCl_2$ for 3 h. Radioactivity released into the supernatant was determined with a gamma counter. Data shown are means of duplicates.

excess of either aFGF or bFGF. Heparin binds to angiogenin [Soncin et al., 1997] and appears to be an important regulator of angiogenesis. At concentrations of 10 and 100 µg/ml, it inhibits nuclear translocation of angiogenin by 10% and 37%, respectively.

Dissociation of ^{125}I-Angiogenin From the Nucleus

Nuclear-bound angiogenin undergoes little spontaneous dissociation. No appreciable amount of ^{125}I-angiogenin is released from isolated nuclei during 1-h incubation in PBS either in the absence or in the presence of 10 µg/ml unlabeled angiogenin or RNase A (Table VI). Incubation for 3 and 24 h results in the release of only about 5% and 40% of the nuclear ^{125}I-angiogenin, respectively, under all three

conditions. This finding indicates that angiogenin is tightly bound in the nucleus and that the binding molecule is not RNA. Nuclear-bound ^{125}I-angiogenin is released by DNase I in both a time-dependent (Table VI) and dose-dependent (Fig. 4) manner. A significant amount of angiogenin (870 cpm, 21% of total) is already released by 1-h incubation with 10 µg/ml DNase I. Increasing the incubation time to 2 and 3 h results in the release of 1,350 and 1,530 cpm ^{125}I-angiogenin from the nuclei, respectively, that is, 33% and 37% of the total nuclear ^{125}I-angiogenin. Complete dissociation occurs with 24-h incubation. Specific release of nuclear ^{125}I-angiogenin by DNase I indicates that angiogenin binds to DNA in the nucleus. However, the nonlinear relationships between angiogenin release and incubation time or DNase I concentration suggest that the binding sites for angiogenin in the nucleus are heterogeneous.

Surprisingly, micrococcal nuclease failed to release ^{125}I-angiogenin from the nucleus. To confirm that micrococcal nuclease digested DNA of the isolated nuclei, the DNA was metabolically labeled with ^{3}H-thymidine before ^{125}I-angiogenin was added to allow nuclear translocation to take place. Table VII shows that unlabeled angiogenin and RNase A release neither ^{125}I-angiogenin nor ^{3}H-thymidine, as expected. However, whereas both DNase I and micrococcal nuclease release ^{3}H-thymidine, only DNase I releases ^{125}I-angiogenin. It is unknown why micrococcal nuclease fails to release angiogenin from the nucleus.

DISCUSSION

Nuclear translocation of polypeptide growth factors in their target cells [Levine and Prystowsky, 1995] has been recognized and termed the "third messenger" [Laduron, 1994] that pro-

**TABLE VII. Release of ^{125}I-Angiogenin and
[3]H-Thymidine From Nucleus***

Enzymes	Released ^{125}I-angiogenin (cpm)	Released ^{3}H-thymidine (cpm)
PBS	100 ± 10	100 ± 10
RNase A (10 µg/ml)	110 ± 10	110 ± 10
Angiogenin (10 µg/ml)	80 ± 10	110 ± 10
DNase I (10 µg/ml)	710 ± 40	5,600 ± 260
Micrococcal nuclease (10 µg/ml)	150 ± 10	3,140 ± 170

PBS, phosphate-buffered saline; HUVE, human umbilical vein endothelial.

*HUVE cells, 50,000 per 35-mm dish, were metabolically labeled with 1 µCi of ^{3}H-thymidine for 24 h. The cells were washed three times with HE-SFM and incubated with 200 ng/ml of ^{125}I-angiogenin for 30 min. The nuclear fraction was isolated and incubated with different enzymes at room temperature for 6 h in PBS containing 5 mM $CaCl_2$ and 10 mM $MgCl_2$. One-half of the supernatant was used to determine ^{125}I with a gamma counter; the other half was used to determine ^{3}H by liquid scintillation counting. Data shown are means and ranges of duplicates of each sample in a representative experiment.

vides specificity to polypeptide-induced gene activation and transcription events [Jans, 1994]. More than 20 growth factors, cytokines, and hormones have been reported to undergo nuclear translocation. Among them, certain angiogenic polypeptides constitute a unique group in that nuclear translocation in endothelial cells appears to be essential for their angiogenic activities [Moroianu and Riordan, 1994b]. Angiogenin is particularly interesting in this regard, since its activity depends both on nuclear translocation and on its ribonucleolytic activity. Presumably, angiogenin elicits its ribonucleolytic action only after it reaches the cell nucleus. Compounds that inhibit either the nuclear translocation process or the ribonucleolytic activity but not both have been shown to abolish its angiogenic activity [Hu, 1998; Nobile et al., 1998]. An inhibitor of nuclear translocation has a distinct advantage in controlling unwanted angiogenesis. Because it targets an intracellular action of angiogenin in endothelial cells, it would provide both the sensitivity and the selectivity needed to make an angiogenesis inhibitor suitable for clinical use. It would be more effective than a direct angiogenin antagonist would be because it obviates neutralization of all circulating angiogenin. However, since it would only inhibit induced proliferation of endothelial cells without any significant effect on basal level and spontaneous proliferation, it would have greater selectivity than would more general inhibitors of endothelial cell growth. Understanding the details of nuclear translocation would be critical for designing inhibitors of this process or for modifying existing ones. The results reported in this article indicate a fascinating process used by angiogenin for its nuclear translocation.

Nuclear translocation of angiogenin is remarkably fast. It takes only 2 min for exogenous angiogenin to appear in the nucleus of HUVE cells. Among the growth factors and cytokines reported to undergo nuclear translocation, only growth hormone does so with a rate comparable to that of angiogenin [Lobie et al., 1994a]. The rapid accumulation of angiogenin in the endothelial cell nucleus suggests that angiogenin is able to evoke its nuclear function within a few minutes. Hormones and growth factors elicit acute responses that occur within seconds after binding to their receptor, such as activation of the tyrosine kinase activity of the receptor. However, hormones and growth factors also elicit much slower responses that occur after many hours, such as the initiation of DNA synthesis. Activation of early-response genes such as c-fos, c-jun, and c-myc occurs within the first 30 min of exposure to growth factors. Fast translocation of angiogenin would ensure its availability in the nucleus for such responses. As an angiogenic protein whose ribonucleolytic activity is necessary and seems to act in the cell nucleus, a fast translocation would be more advantageous than the slow translocation used by other growth factors. For example, ^{125}I-epidermal growth factor (EGF) was reported to accumulate in the nuclei of bovine corneal endothelial cells only after a lag period of 4 h, taking 24 h to reach saturation [Savion et al., 1981]. ^{125}I-Prolactin was found in the nucleus of rat Nb2 node lymphoma cells only after 3-h incubation at 37°C [Rao et al., 1995]. The mechanism by which angiogenin is translocated to the nucleus might, therefore, be different from that used by other proteins. Nuclear translocation of angiogenin has been demonstrated to be independent of lysosomes and microtubules [Li et al., 1997]. However, lysosome inhibitors increased the accumulation of nuclear ^{125}I-EGF up to 25-fold [Savion et al., 1981]. That neither bFGF nor aFGF inhib-

ited nuclear translocation of ^{125}I-angiogenin also suggests a unique pathway only for angiogenin.

Another remarkable finding is that up to 85% of the cell-associated angiogenin is in the nucleus of HUVE cells. This percentage of nuclear angiogenin is much higher than that found for prolactin or bFGF. Thus, only 30% of internalized and 5% of total cell-associated prolactin is in the nucleus of node lymphoma cells after 3 h [Rao et al., 1995] and no more than 40–50% of the total internalized bFGF was in the nucleus of venular endothelial cells after 8 h [Hawker and Granger, 1992]. That most of the internalized angiogenin is in the nucleus, rather than in the cytosol, also implies an important function of nuclear angiogenin. Data on subcellular distribution indicate that transportation across the cytoplasm occurs via a vesicular route that avoids any unnecessary contact between angiogenin and the cytosolic ribonuclease inhibitor.

Translocation of angiogenin reaches saturation in 15 min and the amount within the nucleus remains unchanged for at least 4 h. During this period, no increase in cytosolic angiogenin is observed. Therefore, when the nucleus is saturated with angiogenin, the cell either needs to shut down its translocation machinery to prevent any new angiogenin from being taken up, or it must actively transport nuclear angiogenin out of the cells to maintain such a balance. Given the lack of spontaneous dissociation of angiogenin from the nuclear fraction, the first possibility seems more likely.

Cell density is the most important factor for the capacity of cells to translocate angiogenin into the nucleus. Previous studies have shown that nuclear angiogenin was detected only in proliferating endothelial cells [Moroianu and Riordan, 1994a]. In agreement with these results, we observed that nuclear translocation of ^{125}I-angiogenin decreased progressively with increasing cell density. The positive correlation between the ability of cells to translocate angiogenin to the nucleus and the expression of a cell surface receptor for angiogenin implies a role for the receptor in the nuclear translocation process. A receptor-mediated endosomal route has been postulated for nuclear translocation of a number of polypeptides including insulin, interleukin-1α, EGF, prolactin, and growth hormone [Lobie et al., 1994a]. However, it seems that only early endosomes are involved in the translocation of angiogenin, since late endosomes usually fuse with lysosomes and their transportation requires a microtubule network [Matteoni and Kreis, 1987]. The fact that microtubule-disrupting agents and lysosomal inhibitors do not affect nuclear translocation of angiogenin suggests that the route for angiogenin diverges from that for other polypeptides at the point of early endosomes.

Receptors for multiple polypeptide ligands have been reported to be nuclear-associated, including growth hormone, insulin, prolactin, angiotensin II, nerve growth factor, platelet-derived growth factor, EGF, and FGF [Lobie et al., 1994b]. We do not know whether the angiogenin receptor is co-translocated to the nucleus with angiogenin. Heparin, a molecule to which many angiogenic proteins bind, inhibits nuclear translocation of angiogenin in a seemingly dose-dependent manner. However, it is unknown how this inhibition occurs and whether heparin interferes with the binding of angiogenin to its receptor. Angiogenin has been shown to mediate cell-adhesion of both tumor and endothelial cells via a cell-surface proteoglycan. However, this proteoglycan does not appear to be the same as the receptor responsible for cell proliferation. Moreover, angiogenin-mediated cell adhesion does not require nuclear translocation of angiogenin.

Polypeptides in the nucleus have been thought to provide the necessary specificity of transcriptional events in a manner analogous to steroid hormone-induced transcription, where the receptor for the steroid hormone is an integral component of the transcription complex [Beato, 1989]. Kinase activation in the target cells does not lend specificity, even if necessary for signal transduction. A mutant aFGF with deletion in its NLS failed to stimulate DNA synthesis and cell proliferation in vitro, although it could still bind to the FGF receptor and induce intracellular receptor-mediated tyrosine phosphorylation and c-fos expression [Lin et al., 1996]. Mitogenic activity was regenerated by attaching an NLS from histone 2B at the N-terminus such that the chimeric protein could undergo nuclear translocation. Nuclear angiogenin is critical for angiogenin-induced cell proliferation and angiogenesis. However, how it acts and where it binds in the nucleus remain unclear. The release of nuclear angiogenin by DNase I indicates that angiogenin can

bind to DNA in the nucleus. However, it is unclear whether angiogenin acts directly on DNA to regulate gene transcription, although it is possible to envisage such a mechanism that might be coupled to the characteristic ribonucleolytic activity of angiogenin that leads to processing of the transcribed RNA.

REFERENCES

Badet J, Soncin F, Guitton J-D, Lamare O, Cartwright T, Barritault D. 1989. Specific binding of angiogenin to calf pulmonary artery endothelial cells. Proc Natl Acad Sci USA 86:8427–8431.

Barton DPJ, Cai A, Wendt K, Young M, Gamero A, Decesare S. 1997. Angiogenic protein expression in advanced epithelial ovarian cancer. Clin Cancer Res 3:1579–1586.

Beato, M. 1989. Gene regulation by steroid hormones. Cell 56:335–344.

Bläser J, Triebel S, Kopp C, Tschesche H. 1993. A highly sensitive immunoenzymometric assay for the determination of angiogenin. Eur J Clin Chem Clin Biochem 31:513–516.

Bouche G, Gas N, Prats H, Baldin V, Tauber J-P, Teissie J, Amalric F. 1987. Basic fibroblast growth factor enters the nucleolus and stimulates the transcription of ribosomal genes in AGAE cells undergoing $G_0 \rightarrow G_1$ transition. Proc Natl Acad Sci USA 84:6770–6774.

Bussolino F, Mantovani A, Persico G. 1997. Molecular mechanisms of blood vessel formation. TIBS 22:251–256.

Chopra V, Dinh TV, Hannigan EV. 1997. Serum levels of interleukins, growth factors and angiogenin in patients with endometrial cancer. J Cancer Res Clin Oncol 123:167–172.

Chopra V, Dinh TV, Hannigan EV. 1998. Circulating serum levels of cytokines and angiogenic factors in patient with cervical cancer. Cancer Invest 16:152–159.

Hanahan D, Folkman J. 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86:353–364.

Hawker JR Jr, Granger HJ. 1992. Internalized basic fibroblast growth factor translocates to nuclei of venular endothelial cells. Am J Physiol 262:H1525–1537.

Hu G-F. 1998. Neomycin inhibits angiogenin-induced angiogenesis. Proc Natl Acad Sci USA 95:9791–9795.

Hu G-F, Riordan JF. 1993. Angiogenin enhances actin acceleration of plasminogen activation. Biochem Biophys Res Commun 197:682–687.

Hu G-F, Strydom DJ, Fett JW, Riordan JF, Vallee BL. 1993. Actin is a binding protein for angiogenin. Proc Natl Acad Sci USA 90:1217–1221.

Hu G-F, Riordan JF, Vallee BL. 1994. Angiogenin promotes invasiveness of cultured endothelial cells by stimulation of cell-associated proteolytic activities. Proc Natl Acad Sci USA 91:12096–12100.

Hu G-F, Riordan JF, Vallee BL. 1997. A putative angiogenin receptor in angiogenin-responsive human endothelial cells. Proc Natl Acad Sci USA 94:2204–2209.

Jans DA, 1994. Nuclear signaling pathways for polypeptide ligands and their membrane receptors? FASEB J 8:841–847.

Jimi S-I, Ito K-I, Kohno K, Ono M, Kuwano M, Itagaki Y, Isikawa H. 1995. Modulation by bovine angiogenin of tubular morphogenesis and expression of plasminogen activator in bovine endothelial cells. Biochem Biophys Res Commun 211:476–483.

Laduron PM. 1994. From receptor internalization to nuclear translocation: new targets for long-term pharmacology. Biochem Pharmacol 47:3–13.

Levine JE, Prystowsky MB. 1995. Polypeptide growth factors in the nucleus: a review of function and translocation. Neuroimmunomodulation 2:290–298.

Li D, Bell J, Brown A, Berry CL. 1994. The observation of angiogenin and basic fibroblast growth factor gene expression in human colonic adenocarcinomas, gastric adenocarcinomas, and hepatocellular carcinomas. J Pathol 172:171–175.

Li R, Riordan JF, Hu G-F. 1997. Nuclear translocation of human angiogenin in cultured human umbilical artery endothelial cells is microtubule and lysosome independent. Biochem Biophys Res Commun 238:305–312.

Lin Y-Z, Yao SY, Hawiger J. 1996. Role of the nuclear localization sequence in fibroblast growth factor-1-stimulated mitogenic pathways. J Biol Chem 271:5305–5308.

Lobie PE, Mertani H, Morel G, Morales-Bustos O, Norstedt G, Waters MJ. 1994a. Receptor-mediated nuclear translocation of growth hormone. J Biol Chem 269:21330–21339.

Lobie PE, Wood TJJ, Chen CM, Waters MJ, Norstedt G. 1994b. Nuclear translocation and anchorage of the growth hormone receptor. J Biol Chem 269:31735–31746.

Matteoni R, Kreis TE. 1987. Translocation and clustering of endosomes and lysosomes depends on microtubules. J Cell Biol 105:1253–1265.

Moroianu J, Riordan JF. 1994a. Nuclear translocation of angiogenin in proliferating endothelial cells is essential to its angiogenic activity. Proc Natl Acad Sci USA 91:1677–1681.

Moroianu J, Riordan JF. 1994b. Nuclear translocation of angiogenic proteins in endothelial cells: an essential step in angiogenesis. Biochemistry 33:12535–12539.

Moroianu J, Riordan JF. 1994c. Identification of the nucleolar targeting signal of human angiogenin. Biochem Biophys Res Commun 203:1765–1772.

Nobile V, Russo N, Hu G-F, Riordan J F. 1998. Inhibition of human angiogenin by DNA aptamers: Nuclear colocalization of an angiogenin-inhibitor complex. Biochemistry 37:6857–6863.

Rao YP, Buckley DJ, Olson MD, Buckley AR. 1995. Nuclear translocation of prolactin: collaboration of tyrosine kinase and protein kinase C activation in rat Nb2 node lymphoma cells. J Cell Physiol 163:266–276.

Riordan JF. 1997. Angiogenin. In: D'Alessio G, Riordan JF, editors. Ribonucleases: structures and functions. New York: Academic Press. p 459–489.

Savion N, Vlodavsky I, Gospodarowicz D. 1981. Nuclear accumulation of epidermal growth factor in cultured bovine corneal endothelial and granulosa cells. J Biol Chem 256:1149–1154.

Shapiro R, Vallee BL. 1987. Human placental ribonuclease inhibitor abolishes both angiogenic and ribonucleolytic activities of angiogenin. Proc Natl Acad Sci USA 84:2238–2241.

Shapiro R, Riordan JF, Vallee BL. 1986. Characteristic ribonucleolytic activity of human angiogenin. Biochemistry 25:3527–3532.

Shapiro R, Harper W, Fox EA, Jansen H-W, Hein F, Uhlmann E. 1988. Expression of Met-(−1) angiogenin in *Escherichia coli*: conversion to the authentic <Glu-1 protein. Anal Biochem 175:450–461.

Shimoyama S, Gansauge F, Gansauge S, Negri G, Oohara T, Beger HG. 1996. Increased angiogenin expression in pancreatic cancer is related to cancer aggressiveness. Cancer Res 56:2703–2706.

Soncin F. 1993. Angiogenin supports endothelial and fibroblast cells adhesion. Proc Natl Acad Sci USA 89:2232–2236.

Soncin F. Strydom DJ, Shapiro R. 1997. Interaction of heparin with human angiogenin. J Biol Chem 272:9818–9824.

Vasandani VM, Wu Y-N, Mikulski SM, Youle RJ, Sung C. 1996. Molecular determinants in the plasma clearance and tissue distribution of ribonucleases of the ribonuclease A superfamily. Cancer Res 56:4180–4186.

Zhan X, Hu X, Friedman S, Maciag T. 1992. Analysis of endogenous and exogenous nuclear translocation of fibroblast growth factor-1 in NIH 3T3 cells. Biochem Biophys Res Commun 188:982–991.