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Nuclear translocation of angiogenin in proliferating endothelial cells is essential to its angiogenic activity

(angiogenesis/endocytosis/growth factors/immunofluorescence/nucleolus)

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ABSTRACT **The intracellular pathway of human angiogenin in calf pulmonary artery endothelial (CPAE) cells has been studied by immunofluorescence microscopy. Proliferating CPAE cells specifically endocytose native angiogenin and translocate it to the nucleus, where it accumulates in the nucleoli. Nuclear translocation of angiogenin does not occur in nonproliferative, confluent CPAE cells. These cells were previously found to express an angiogenin-binding protein (AngBP) that was identified as smooth muscle α-actin. Exogenous actin, an anti-actin antibody, heparin, and heparinase treatment all inhibit the internalization of angiogenin, suggesting the involvement of cell surface AngBP/actin and heparan sulfate proteoglycans in this process. It has been established that two regions of angiogenin are essential for its angiogenic activity, one is its endothelial cell binding site and the other its catalytic site capable of cleaving RNA. CPAE cells do not internalize four enzymatically active angiogenin derivatives whose cell binding site is modified, but they do internalize two enzymatically inactive mutants whose cell binding site is intact. Thus, the putative cell binding site of angiogenin is necessary for both endocytosis and nuclear translocation, but the catalytic site is not. Three other angiogenic molecules are also translocated to the nucleus of growing CPAE cells. Overall, the results suggest that nuclear translocation of angiogenin and other angiogenic molecules is a critical step in the process of angiogenesis.**

Angiogenin, a 14-kDa potent inducer of angiogenesis (1–3), is a member of the pancreatic RNase superfamily (4) with a unique ribonucleolytic activity (5, 6) that is critical for its angiogenic activity. A region of angiogenin distinct from the catalytic site, defined as the cell binding site and including residues 60–68 (7) and Asn-109 (8), is also necessary for angiogenesis. Angiogenin interacts with endothelial cells via specific receptor(s) to activate their phospholipase pathways (9, 10) and binds specifically to the endothelial cell surface (11). It was shown to bind to a 42-kDa dissociable cell surface component of calf pulmonary artery endothelial (CPAE) and GM7373 cells (12). Tryptic peptide mapping and amino acid sequence analysis identified this angiogenin-binding protein (AngBP) as a member of the muscle-type actin family (13). Immunofluorescent staining demonstrates that a smooth muscle type of α-actin is a surface component of cultured CPAE cells and can interact with angiogenin (14).

Immunofluorescence with an anti-human angiogenin monoclonal antibody (mAb) has now identified an intracellular pathway for human angiogenin in growing CPAE cells. The protein is endocytosed and translocated from the cell surface to the nucleus, where it accumulates in the nucleoli. The use of various angiogenin mutants has established that the cell binding site of angiogenin is critical for internalization and nuclear translocation but the catalytic site is not, suggesting that angiogenin might act as a RNase within the nucleolus.

Previous studies reported the accumulation of other angiogenic factors in the nucleus of target cells. Thus, exogenous basic fibroblast growth factor (bFGF), acidic FGF (aFGF), and epidermal growth factor (EGF) all undergo nuclear translocation (15–18). More recently, nuclear targeting sequences were identified in bFGF (19) and aFGF (20). Our investigation of the nuclear translocation of angiogenin also includes the intracellular pathway of these other angiogenic molecules, and we find that proliferating CPAE cells translocate all of them from the cell surface to the nucleus. Nuclear translocation of angiogenic molecules therefore appears to be a general pathway that is essential to the mechanism of angiogenesis.

MATERIALS AND METHODS

Reagents. Human Met-(−1)-angiogenin and plasma angiogenin were obtained as described (2, 21). Angiogenin K, a proteolytic derivative of Met-(−1)-angiogenin cleaved at residues 60–61 (7), was provided by T. W. Hallahan. ARH-I is a Met-(−1)-angiogenin/RNase hybrid protein in which the angiogenin segment 58–70 has been replaced by residues 59–73 of RNase (22). The site-specific mutants R66A (Arg-66 → Ala), R33A, N109D, H13A, and K40Q were prepared as described (8, 23–25) and provided by R. Shapiro. An IgG1(κ) mAb to human angiogenin, designated as 26-2F, was provided by K. A. Olson (Harvard Medical School). This mAb recognizes human angiogenin and the mutants used in this study but does not react with bovine angiogenin, as determined by ELISA and RIA (K. A. Olson and J. W. Fett, personal communication). Bovine bFGF, bovine aFGF, anti-bFGF IgG, anti-aFGF IgG, a mAb to smooth muscle α-actin, and heparin (from porcine intestinal mucosa) were from Sigma. Fluorescein isothiocyanate (FITC)-labeled goat $F(ab')_2$ anti-mouse IgG and FITC-labeled goat $F(ab')_2$ anti-rabbit IgG were from Caltag (South San Francisco, CA). Nonimmune mouse IgG1(κ) was from Organon Teknika-Cappel. EGF Bodipy FL conjugate and acetylated low density lipoprotein Bodipy complex were from Molecular Probes. A mAb to the proliferating-cell nuclear antigen (PCNA) was from Oncogene Science.

Cell Culture. CPAE cells (CCL209) from the American Type Culture Collection were obtained at passage 19 and used until passage 26 and were cultured in minimal essential medium (MEM) containing 20% heat-inactivated fetal bovine

Abbreviations: ARH-I, Met-(−1)-angiogenin in which residues 58–70 have been replaced by residues 59–73 of RNase A; AngBP, angiogenin-binding protein; ABAE, adult bovine aortic endothelial; bFGF, basic fibroblast growth factor; aFGF, acidic FGF; CAM, chorioallantoic membrane; CPAE, calf pulmonary artery endothelial; NLS, nuclear localization signal; PCNA, proliferating-cell nuclear antigen; EGF, epidermal growth factor; mAb, monoclonal antibody.

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serum (FBS) and antibiotics (11). BALB/c 3T3 fibroblasts (from J. W. Fett, Harvard Medical School) and GM7373 transformed bovine endothelial cells (Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ) were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS and antibiotics.

Fluorescence Microscopy. Cells were observed with a Nikon Labophot fluorescence microscope. Photomicrographs were taken with an advanced UFX camera system and TMY 400 film at an exposure setting of 400. In general, photographs depicting the absence of staining or results that are indistinguishable from those of a positive control are not presented.

Immunofluorescence for Intracellular Localization of Angiogenic Factors and Cell Proliferation Status. CPAE cells were grown on glass coverslips for 1 day (subconfluent cells) or for 3 days (confluent cells). Subconfluent cells were washed three times with serum-free MEM and then incubated with human angiogenin (1 μg/ml) in MEM for 30 min at 37°C or with bFGF or aFGF (50 ng/ml) for 2 hr at 37°C. Confluent cells were washed three times with MEM and kept in MEM for 24 hr prior to incubation. Cell viability, determined after this treatment by trypan blue exclusion, was >95%. After incubation cells were washed three times in MEM at 4°C and fixed and permeabilized with methanol for 10 min at −20°C. Fixed cells were rinsed three times with 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and incubated with anti-human angiogenin mAb 26-2F (5 μg/ml) for 1 hr at 37°C. Cells treated with bFGF and aFGF were incubated with anti-bFGF IgG (100 μg/ml in BSA/PBS) or anti-aFGF IgG (50 μg/ml in BSA/PBS), respectively, for 1 hr at 37°C. They were then washed five times with BSA/PBS followed by addition of FITC-labeled goat $F(ab')_2$ anti-mouse or anti-rabbit IgG (1:100 dilution). After 1 hr, the cells were washed five times with BSA/PBS and once with PBS and mounted in glycerol/PBS (1:1, vol/vol). Controls for cells treated with angiogenic factors were (*a*) omission of the first antibody and (*b*) substitution of the first antibody by nonimmune mouse or rabbit IgG at the same concentration. Cells not treated with angiogenic factors did not stain with their specific antibodies. In some experiments angiogenin mutants were substituted for the native protein. CPAE cells were immunostained (26) with a mAb to PCNA.

RESULTS AND DISCUSSION

Nuclear Localization of Angiogenin in CPAE Cells. Growing CPAE cells (1 day) were incubated with human Met-(−1)-angiogenin (1 μg/ml) for 30 min at 37°C and examined for its intracellular localization by immunostaining with mAb 26-2F. Bright staining was observed in the nucleoli with lower intensity staining in the nucleoplasmic network (Fig. 1*a*). Native <Glu-angiogenin also underwent nuclear localization. Nucleolar localization could be detected readily with angiogenin at 1 μg/ml (Fig. 1*a*) or 100 ng/ml but only marginally with 50 ng/ml (data not shown). Staining was not observed in control cells which were not exposed to angiogenin or when angiogenin-treated cells were exposed to a nonimmune mouse IgG. PCNA, a nuclear marker characteristic of proliferating cells, was detected in the nucleus of the subconfluent CPAE cells by immunostaining (Fig. 1*b*). Neither lysozyme (like angiogenin, a strongly basic protein) nor RNase A (which has 35% sequence identity with angiogenin) affected internalization or nuclear translocation of angiogenin in CPAE cells. Internalization was abolished by a 10-fold molar excess of bovine angiogenin as revealed by immunostaining with mAb 26-2F (which does not react with bovine angiogenin). Fluorescently labeled acetylated low density lipoprotein, a conventional endothelial cell marker that localizes to lysosomes, was used to ensure the specificity of nuclear translocation of angiogenin. As expected, it accumulated in cytoplasmic lysosomes, not in the nucleus (Fig. 2). Angiogenin was also internalized and translocated to the nucleus in growing GM7373 endothelial cells but not in BALB/c 3T3 fibroblasts.

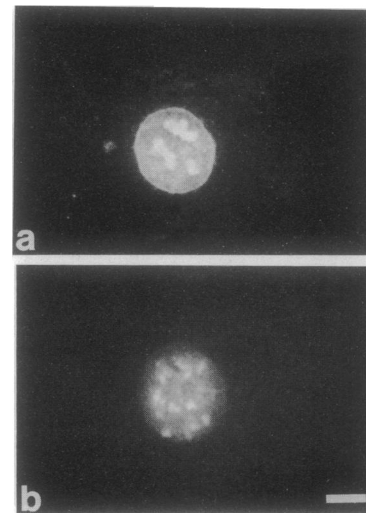

FIG. 1. (*a*) Nuclear localization of internalized human angiogenin in proliferating CPAE cells revealed by immunofluorescence. Cells were incubated with human angiogenin (1 μg/ml) for 30 min at 37°C, washed in MEM, fixed with methanol, and immunostained with mAb 26-2F (5 μg/ml). (*b*) Nuclear immunostaining of CPAE cells with an anti-PCNA mAb. (Bar = 10 μm.)

Kinetics of Angiogenin Appearance in the Nucleus and Nucleolus of CPAE Cells. After 5 min of incubation, angiogenin was detected primarily in the cytoplasm (Fig. 3*a*), after 15 min it was found largely in the nucleus (Fig. 3*b*), and preferential accumulation in the nucleoli was observed after 60 min (Fig. 3*c*). As shown in Fig. 1*a*, both the nucleolus and the nucleoplasm were immunostained at 30 min. Transport of angiogenin to the nucleus and nucleolus is therefore very rapid, as reported for bFGF (15, 16).

Angiogenin Internalization Occurs by Endocytosis. The specific binding of angiogenin to CPAE cells (11, 12) and its subsequent internalization indicate that it most likely undergoes endocytosis. To examine this, subconfluent cells were incubated at 4°C with angiogenin (1 μg/ml) for either 30 min or 2 hr. The cells were washed with cold MEM, and the intracellular localization of angiogenin was examined by immunofluorescence. No angiogenin could be detected in the nucleus of these cells. Endocytosis requires ATP and is blocked by inhibition of both glycolysis and oxidative phosphorylation. Therefore, before exposure to angiogenin the CPAE cells were preincubated at 37°C for 2 hr with 50 mM 2-deoxyglucose, an inhibitor of glycolysis, and with 10 mM NaN_3, an inhibitor of oxidative phosphorylation (27). This pretreatment did not alter cell viability. No intracellular angiogenin could be detected.

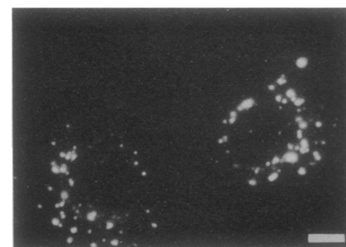

FIG. 2. Cytoplasmic localization of the fluorescent acetylated low density lipoprotein Bodipy conjugate internalized by incubation (10 μg/ml) with CPAE cells for 4 hr at 37°C. (Bar = 10 μm.)

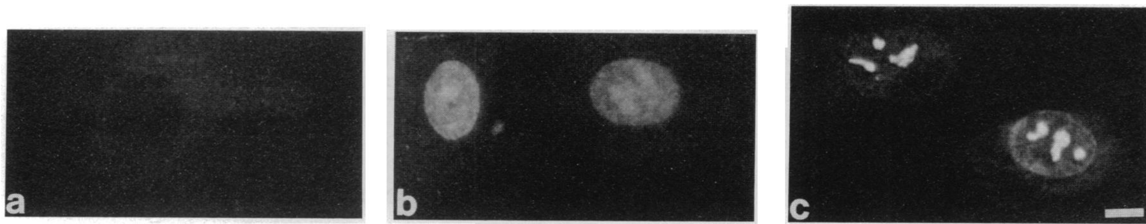

FIG. 3. Kinetics of angiogenin translocation to the nuclei in proliferating CPAE cells revealed by immunofluorescence. Cells were incubated with angiogenin (1 μg/ml) for 5 min (*a*), 15 min (*b*), or 60 min (*c*) and immunostained with mAb 26-2F. (Bar = 10 μm.)

Angiogenin Uptake and Nuclear Translocation Are Related to Cell Proliferation. In contrast with proliferating cells, confluent nonproliferating CPAE cells do not internalize angiogenin. Even after 24 hr, only very weak immunofluorescence could be detected in the cytoplasm (data not shown). The nonproliferative state of these confluent cells was confirmed by their failure to stain with the PCNA mAb. The absence of nuclear translocation suggests a direct relationship between this process and the proliferative state of the cell, most likely reflecting the decreased number of angiogenin binding sites on the surface of confluent CPAE cells (11) and perhaps a specific nuclear translocation mechanism that operates only in proliferating cells, as for bFGF in adult bovine aortic endothelial (ABAE) cells (16).

Exogenous Actin and Heparin Inhibit Internalization of Angiogenin by CPAE Cells. An AngBP isolated previously from endothelial cells is a member of the actin family (12, 13). Moreover, a smooth muscle type of α-actin was found on the surface of CPAE cells, where it interacted with angiogenin (14). We therefore investigated whether binding of angiogenin to cell surface actin was required for the internalization process. Subconfluent CPAE cells were incubated with angiogenin in the presence or absence of actin and immunostained with mAb 26-2F. With actin at 1 or 10 μg/ml, angiogenin was not detected in CPAE cells. A mAb to smooth muscle α-actin (100 μg/ml) also inhibited internalization, thus indicating that internalization could involve binding to cell surface AngBP/actin followed by endocytosis.

Heparin (10, 1, and 0.1 μg/ml) inhibited the internalization of angiogenin in CPAE cells but chondroitin sulfate did not. Treatment of the cells with heparinase (28) also markedly reduced the internalization of angiogenin (data not shown). Moreover, an 8-fold molar excess of bFGF [known to bind to surface heparan sulfates (28)] completely inhibited angiogenin endocytosis. Thus it appears that in addition to AngBP, cell surface heparan sulfate proteoglycans may also be involved in the internalization of angiogenin.

Specificity of Internalization and Nuclear Translocation of Angiogenin Variants in Endothelial Cells. Two distinct regions of angiogenin are essential for its angiogenic activity: a catalytic site containing His-13, Lys-40, and His-114 that is capable of cleaving RNA (23, 24) and a noncatalytic, cell binding site encompassing minimally residues 60–68 (7) plus a segment containing Asn-109 (8). The importance of each of the two sites for internalization and nuclear translocation of angiogenin was examined with six angiogenin variants modified at either the putative cell binding site or the catalytic site but which are still recognized in RIA and ELISA by mAb 26-2F (K. A. Olson and J. W. Fett, personal communication). The four enzymatically active angiogenin variants in which the cell binding site is altered—angiogenin K, ARH-I, R66A, and N109D—were tested under the same incubation conditions as those for human angiogenin. None of them could be detected in the nucleus of CPAE cells, unlike native angiogenin, for which there was strong nuclear staining (Fig. 4*a*). Thus, the putative cell binding site is critical for internalization of angiogenin by CPAE or GM7373 cells (Table 1), and specific receptor-mediated endocytosis is part of the process of nuclear translocation. Moreover, N109D, which competes with the native protein in the CAM assay and is fully active enzymatically (8), is neither translocated to the nucleus nor angiogenic (Table 1). Hence, ribonucleolytic activity and receptor binding capacity, while necessary, are not sufficient for angiogenic activity: endocytosis and nuclear translocation are required as well.

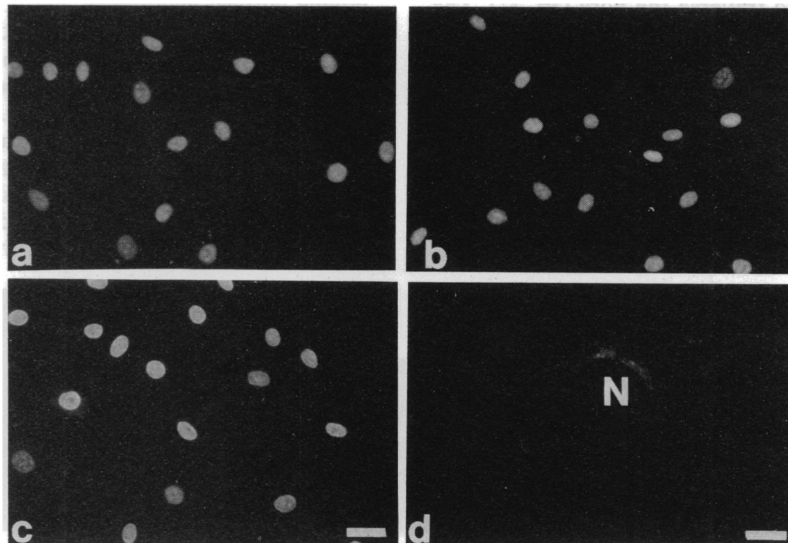

FIG. 4. Nuclear immunolocalization of human angiogenin (*a*) or mutants H13A (*b*) and K40Q (*c*) (each at 1 μg/ml) in proliferating CPAE cells revealed by immunostaining with mAb 26-2F. (Bar = 40 μm.) (*d*) Perinuclear immunolocalization of R33A in growing CPAE cells. N, nucleus. (Bar = 10 μm.)

Table 1. Activities and CPAE and GM7373 cells nuclear localization of angiogenin derivatives

Mutation	Enzymatic activity,* %	Angiogenic activity†	Competition with angiogenin‡	Nuclear localization§
None	100	+	+	+
Angiogenin K	100	−	−	−
ARH-I	30,000	−	−	−
R66A	76	−	ND	−
N109D	100	−	+	−
K40Q	<0.05	−	ND	+
H13A	<0.01	−	+	+

*Measured with tRNA as substrate, at pH 6.8 (7, 8, 22–25).
†Measured by chorioallantoic membrane (CAM) assay (7, 8, 22–25).
‡In CAM assay, as reported previously (7, 8, 22–25). ND, not determined.
§Determined by immunostaining with mAb 26-2F, which recognizes all derivatives used in ELISA and RIA (K. A. Olson and J. W. Fett, personal communication). +, Nuclear immunostaining comparable with the positive control (native angiogenin).

In contrast, two enzymatically inactive mutants, K40Q and H13A, whose cell binding site was intact were translocated to the nucleoli of proliferating CPAE cells, much like native angiogenin (Fig. 4 *a–c*). Immunodetection was possible even at 50 ng/ml (data not shown). Moreover, as with native angiogenin, these mutants were not detected in the nucleoli of confluent, nonproliferative CPAE cells. Clearly, the catalytic site of angiogenin is not required for translocation. Since ribonucleolytic activity is critical for angiogenic activity, it seems reasonable to conclude that the putative substrate(s) for angiogenin *in vivo* (as yet unidentified) must be localized in the nucleus, most likely at the nucleolar site where rRNA synthesis and processing take place.

Proteins that are translocated to the nucleus of eukaryotic cells are targeted by specific nuclear localization signals (NLSs) that typically contain a high proportion of basic amino acids (29). The sequence Arg^{31}-Arg^{32}-Arg^{33} in human angiogenin (4) might constitute part of such a NLS and be involved in nuclear translocation. We examined the mutant R33A, which lacks angiogenic activity (25) but has an intact cell binding site and exhibits 17% enzymatic activity toward tRNA (25). Although some weak perinuclear staining was detected, R33A did not undergo nuclear localization (Fig. 4*d*), suggesting that Arg-33 could be a component of a NLS. If angiogenin follows the nuclear pore route for delivery into the nucleus it must first be released from the endocytotic vesicle into the perinuclear region in order to interact with its NLS-binding protein(s) and traverse the pore (30, 31). The pathway for nuclear and nucleolar translocation of angiogenin, as well as its longevity once inside the nucleus, remains to be established.

Nuclear Translocation of Other Angiogenic Molecules in Proliferating CPAE Cells. Other angiogenic molecules were also examined for their capacity to undergo nuclear translocation in growing CPAE cells. After a 2-hr incubation with bFGF or aFGF (50 ng/ml), both angiogenic factors could be detected predominantly in the nucleoli by immunostaining with the corresponding antibodies (Fig. 5 *a* and *b*). No staining was observed with proliferating cells treated with bFGF or aFGF and exposed to nonimmune rabbit IgG, with cells that were not treated, or with confluent, nonproliferative cells treated with FGFs. Previously, exogenous bFGF was shown to be internalized and specifically translocated to the nucleolus in proliferating but not in confluent ABAE cells (15, 16). Several studies have found that endothelial cells from large vessels and capillaries synthesize bFGF (32–34). However, in CPAE and ABAE cells (15, 16), the amount of endogenous bFGF is too low to be detected by immunostaining with an anti-bFGF antibody. Endogenous bFGF, particularly the 22- and 24-kDa forms, is associated with the nuclei of endothelial cells (35, 36). The terminal extension of these large forms of bFGF contains a NLS (19). In contrast, 18-kDa bFGF accumulates in the nucleus of target cells only when it is provided to the cells exogenously, suggesting the existence of different pathways for nuclear translocation depending on the type of bFGF (37). It is thought that residues 27–32 (Lys-Asp-Pro-Lys-Arg-Leu) of 18-kDa bFGF contain a NLS (38). This sequence is similar to that of residues 23–28 of aFGF, which has also been proposed as a NLS (20).

The EGF Bodipy FL conjugate was also translocated to the nucleus when incubated with proliferating CPAE cells (Fig. 5*c*). Again, there was no detectable fluorescent EGF signal in the nuclei of confluent cells. EGF accumulates in the nuclei of human colorectal carcinoma cells (39, 40) and cultured bovine corneal endothelial and granulosa cells (18). Nerve growth factor, interleukin 1, and platelet-derived growth factor (PDGF) have also been found to be translocated to the nucleus of cells bearing their respective receptors (41), and the exon coding for the NLS of the A chain of PDGF has been identified (42). Thus, nuclear translocation of angiogenic factors (and other growth factors) after endocytosis seems to be a specific and major intracellular pathway and suggests that these factors might function directly in nuclear events. This view is strengthened by the observation (data not

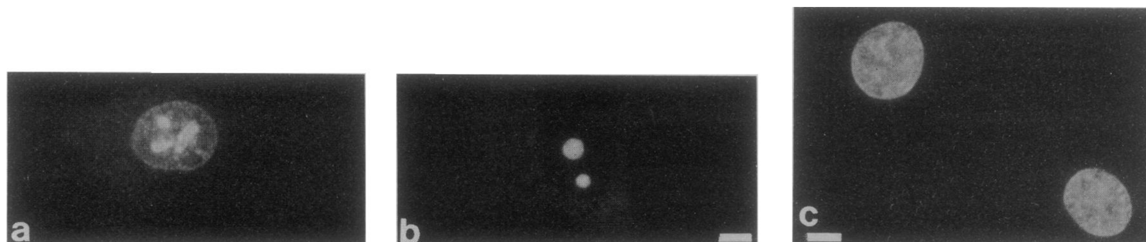

FIG. 5. Nuclear localization of exogenous bFGF, aFGF, and EGF in proliferating CPAE cells. (*a* and *b*) The cells were incubated with bFGF (50 ng/ml) (*a*) or aFGF (50 ng/ml) (*b*) for 2 hr at 37°C and immunostained with anti-bFGF antibody (100 μg/ml) or anti-aFGF antibody (50 μg/ml), respectively. (Bar = 10 μm.) (*c*) Cells were incubated with EGF Bodipy FL conjugate (5 μg/ml) in MEM for 2 hr at 37°C, washed three times in MEM, fixed in phosphate-buffered 3.7% formalin, and examined for fluorescence. (Bar = 10 μm.)

shown) that endocytosis and nuclear translocation of angiogenin, bFGF, and aFGF in proliferating CPAE cells are inhibited by two potent inhibitors of angiogenesis, protamine (43) and platelet factor 4 (44, 45). Protamine also inhibits the mitogenic activity of both bFGF and aFGF (46).

Nuclear Translocation as a General Pathway for Angiogenic Factors. Is nuclear translocation critical to angiogenesis? Angiogenesis occurs by formation of new capillaries from established blood vessels under the action of a variety of angiogenic factors (1, 47). Generally, it involves activation of endothelial cells and degradation of the extracellular matrix, followed by migration, proliferation, and differentiation of the endothelial cells into tube-like structures. Mitogenic and other physiological signals induced by angiogenic factors may be transmitted to the nucleus by the action of intracellular messengers and/or the factors may act directly in the cell nucleus.

Nuclear localization of bFGF correlates with stimulation of the transcription of ribosomal genes during the G_0–G_1 transition in ABAE cells. Moreover, bFGF acts directly on isolated nuclei from quiescent sparse endothelial cells to increase RNA polymerase I transcriptional activity by a factor of 5–6 (16). The proliferative state of a cell is closely related to ribosome biogenesis, which involves a series of coordinated nuclear events, including transcription of ribosomal genes. Cell-free transcription can also be regulated directly by bFGF (48). For aFGF, nuclear translocation is necessary for mitogenic activity (20).

The present data extend the previous dual-site model that has been postulated to account for the mechanism of action of angiogenin (7, 8, 23, 24) to include a nuclear targeting site along with a cell binding site and a catalytic site. Indeed, multiple interaction sites are likely to be a common feature of all angiogenic proteins. Angiogenin also has at least two other functions: it induces second-messenger responses in endothelial cells (9, 10) and supports adhesion of these cells onto a plastic surface (49). It will be interesting to determine how signal transduction correlates with nuclear translocation of angiogenin and to characterize the molecular mechanism of angiogenin action within the nucleolus of endothelial cells.

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