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Nuclear Translocation of Human Angiogenin in Cultured Human Umbilical Artery Endothelial Cells Is Microtubule and Lysosome Independent

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Exogenous angiogenin undergoes rapid nuclear translocation in cultured human umbilical artery endothelial cells at 37 °C but not at 4 °C. Treatment of cells with colchicine, nocodazole and taxol, which disrupt the microtubule system, does not affect the nuclear translocation process of angiogenin, suggesting that cells transport internalized angiogenin in a microtubule independent fashion. Lysosomal inhibitors, chloroquine and leupeptin, neither inhibit nor enhance the nuclear translocation of angiogenin, indicating that lysosomal targeting and processing are not required for, and do not compete with, the nuclear translocation. Moreover, treatment of cells with a tyrosine kinase antagonist, genistein, does not change the ability of the cells to translocate angiogenin into the nucleus. We suggest that exogenous angiogenin is translocated to the nucleus by a mechanism that does not require activation of tyrosine kinase, but includes receptor-mediated endocytosis, microtubule and lysosome independent transport across the cytoplasm, and nuclear localization sequence-assisted nuclear import.

Nuclear translocation of polypeptide growth factors in their target cells (1) has been recognized and termed the "third messenger" (2) that provides specificity to polypeptide-induced gene activation and transcription events (3). More than 15 growth factors, cytokines and hormones have been reported to undergo nuclear translocation. Among them, angiogenic polypeptides are particularly interesting and unique. First, they have the same physiological function, i.e., to induce the formation of new blood vessels (4), although they have very diverse biological and biochemical properties. Second, nuclear translocation of these angiogenic proteins in endothelial cells appears to be essential for their angiogenic activities (5). Nuclear translocation of angiogenin, acidic and basic fibroblast growth factors (FGF), epidermal growth factor, and tumor necrosis factor-α have been reported (5, 6).

Angiogenin is a 14 kDa angiogenic protein isolated from the conditioned medium of human colon adenocarcinoma cells HT-29 (7) based on an *in vivo* angiogenesis assay on the chick chorioallantoic membrane (CAM). It is a member of the pancreatic ribonuclease superfamily and is the only member with potent angiogenic activity (8). The ribonucleolytic activity of angiogenin, which is 4 to 6 orders of magnitude less than that of ribonuclease A, is essential for angiogenesis (9), although none of the other ribonucleases is angiogenic.

Angiogenin induces most of the individual features necessary for the formation of new blood vessels. It binds to endothelial cells (10), interacts with cell surface binding proteins (11) and receptors (12), induces second messengers (13, 14), stimulates cell migration and invasion (15), mediates cell adhesion (16), and promotes cell proliferation (12) and differentiation (17). Moreover, angiogenin has been demonstrated to undergo nuclear translocation in proliferating calf pulmonary artery endothelial (CPAE) cells via receptor-mediated endocytosis (18) and nuclear localization sequence (NLS)-assisted nuclear import (19). Importantly, nuclear translocation of angiogenin appears to be necessary for its angiogenic activity: an angiogenin mutant with intact cell binding capacity and ribonucleolytic activity but with an altered NLS does not accumulate in the nucleolus and fails to induce neovascularization

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Abbreviations used: BSA, bovine serum albumin; CAM, chorioallantoic membrane; CPAE, calf pulmonary artery endothelial; DMEM, Dulbecco's modified Eagle's medium; FGF, fibroblast growth factor; HUAE, human umbilical artery endothelial; NLS, nuclear localization sequence; PBS, phosphate-buffered saline.

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(18, 19). Among angiogenic proteins, angiogenin is unique in that it has ribonucleolytic activity, is translocated to the nucleus and accumulates in the nucleolus. Since both the ribonucleolytic activity and the nuclear translocation process are required for its angiogenic activity, it has been postulated that in addition to the signal transduction pathways triggered by binding to the cell surface receptors and the subsequent transcriptional regulations, angiogenin may exert its ribonucleolytic activity in the nucleolus and therefore facilitate ribosomal biogenesis (5).

Nuclear translocation of exogenous angiogenin would require internalization, transport from the cell surface to the perinuclear region, and import into the nucleus. Receptor-mediated endocytosis seems to be involved in internalization (18). The absolute requirement of an NLS for nuclear translocation suggested a classic nuclear pore route for nuclear import (5, 19). However, the molecular mechanism by which the internalized angiogenin is transported across the cytoplasm to the perinuclear region remains unknown. Since the internalized angiogenin is most likely contained in endosomes that are well known to interact with microtubules and fuse with lysosomes (20), we investigated the involvement of the microtubule system and lysosomes in nuclear translocation of angiogenin by treating cells with drugs that disrupt the microtubules and with reagents that inhibit the function of lysosomes. The data presented in this paper indicate that microtubules and lysosomal functions are not involved in nuclear translocation of angiogenin, suggesting a microtubule and lysosome independent pathway to mediate the transport of angiogenin across the cytoplasm.

MATERIALS AND METHODS

Materials. Human angiogenin was a recombinant product isolated from an *Escherichia coli* expression system (21) and was provided by R. Shapiro. The anti-human angiogenin monoclonal antibody (mAb) 26-2F (IgG_{1k}) was provided by K. A. Olson. FITC-labeled goat $F(ab')_2$ anti-mouse and anti-rabbit IgGs were from Caltag; leupeptin, chloroquine and rabbit anti-tubulin IgG were from Sigma; colchicine, nocodazole and taxol were from Fluka; genistein and daidzein were from Indofine; 2-methoxyestradiol was from Aldrich.

Cell culture. Human umbilical artery endothelial (HUAE) cells were purchased from Cell Systems Corp. as primary cultures isolated from normal human umbilical arteries. They were cultured on attachment factor (Cell Systems)-coated flasks in endothelial cell growth medium CS 3.0 (Cell Systems) containing 10% fetal calf serum, at 37 °C under humidified air containing 5% CO_2. Cells between passages 3 and 8 inclusive were used for all experiments.

Nuclear translocation assay. HUAE cells were trypsinized, seeded on attachment factor-coated cover glass placed in 6-well plates at a density of 5,000 cells/cm^2, and cultured at 37 °C for 24-48 hr under 5% CO_2. Cells were washed 3 times with prewarmed Dulbecco's modified Eagle's medium (DMEM) and incubated with angiogenin at concentrations ranging from 100 ng/ml to 2 μg/ml for 30 min at 37 °C. At the end of the incubation, cells were washed 3 times with DMEM at 4 °C, fixed and permeablized with absolute methanol at −20 °C for 10 min. Immunofluorescent staining of nuclear angiogenin was performed as described previously (18). Briefly, fixed cells were washed 3 times with phosphate-buffered saline (PBS) containing 30 mg/ml bovine serum albumin (BSA) for 10 min at 37 °C and incubated with 10 μg/ml of anti-angiogenin mAb in PBS containing 5 mg/ml BSA at 37 °C for 1 hr. The cells were then washed 5 times with PBS + 5 mg/ml BSA at 37 °C and incubated with FITC-labeled goat $F(ab')_2$ anti-mouse IgG at 1:100 dilution in PBS + 5 mg/ml BSA for 1 hr 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.

Drug treatment. HUAE cells cultured on a cover glass as described above were washed 3 times with DMEM and treated with colchicine (20 μg/ml), nocodazole (20 μg/ml), or taxol (50 μg/ml) in DMEM for 2 hr at 37 °C to destroy microtubules. Immunofluorescence with rabbit anti-tubulin IgG and FITC-labeled goat $F(ab')_2$ anti-rabbit IgG was used to examine the integrity of the microtubule system. To inhibit lysosomal functions, the cells were incubated with 25 μg/ml of leupeptin for 16 hr at 37 °C or with 200 μM chloroquine for 1 hr at 37 °C. With chloroquine concentrations higher than 200 μM, the DMEM was supplemented with 50 mM Hepes, pH 7.5, for extra buffering capacity to maintain a stable pH. After treatment, the cells were washed 3 times with DMEM and incubated with 1 μg/ml of angiogenin in the presence of the corresponding drugs for 30 min at 37 °C. Immunofluorescent staining of nuclear angiogenin was performed as described above.

RESULTS

Nuclear translocation of angiogenin in HUAE cells. HUAE cells were incubated with human angiogenin (1 μg/ml) for a period from 0.5 to 120 min at 37 °C, and examined for nuclear angiogenin by immunofluorescent staining. Nuclear angiogenin was not detected at 1 min (Fig. 1A) but was visible after 2 min (Fig. 1B), reached saturation at 15 min (Fig. 1C), and remained unchanged after 120 min incubation (Fig. 1D). No nuclear angiogenin was detected in controls not exposed to exogenous angiogenin or when a non-immune mAb was used with angiogenin-exposed cells (data not shown). When cells were incubated with different concentrations of angiogenin ranging from 50 ng/ml to 2 μg/ml at 37 °C for 30 min, nucleolar accumulation of angiogenin was detectable at 100 ng/ml. No obvious change was observed at angiogenin concentrations between 200 ng/ml and 2 μg/ml. However, no nuclear angiogenin was detected when the angiogenin concentration was < 50 ng/ml even after prolonged incubation (4 hr) (data not shown).

Effect of microtubule-disrupting agents on nuclear translocation of angiogenin. To examine whether the intracellular movement of internalized angiogenin in HUAE cells is mediated by the microtubule system, we incubated the cells at 37 °C for 2 hr with colchicine (20 μg/ml) or nocodazole (20 μg/ml) which depolymerize microtubules, or with taxol (50 μg/ml) which bundles them, and examined the nuclear translocation of angiogenin in these treated cells. The pharmacological activ-

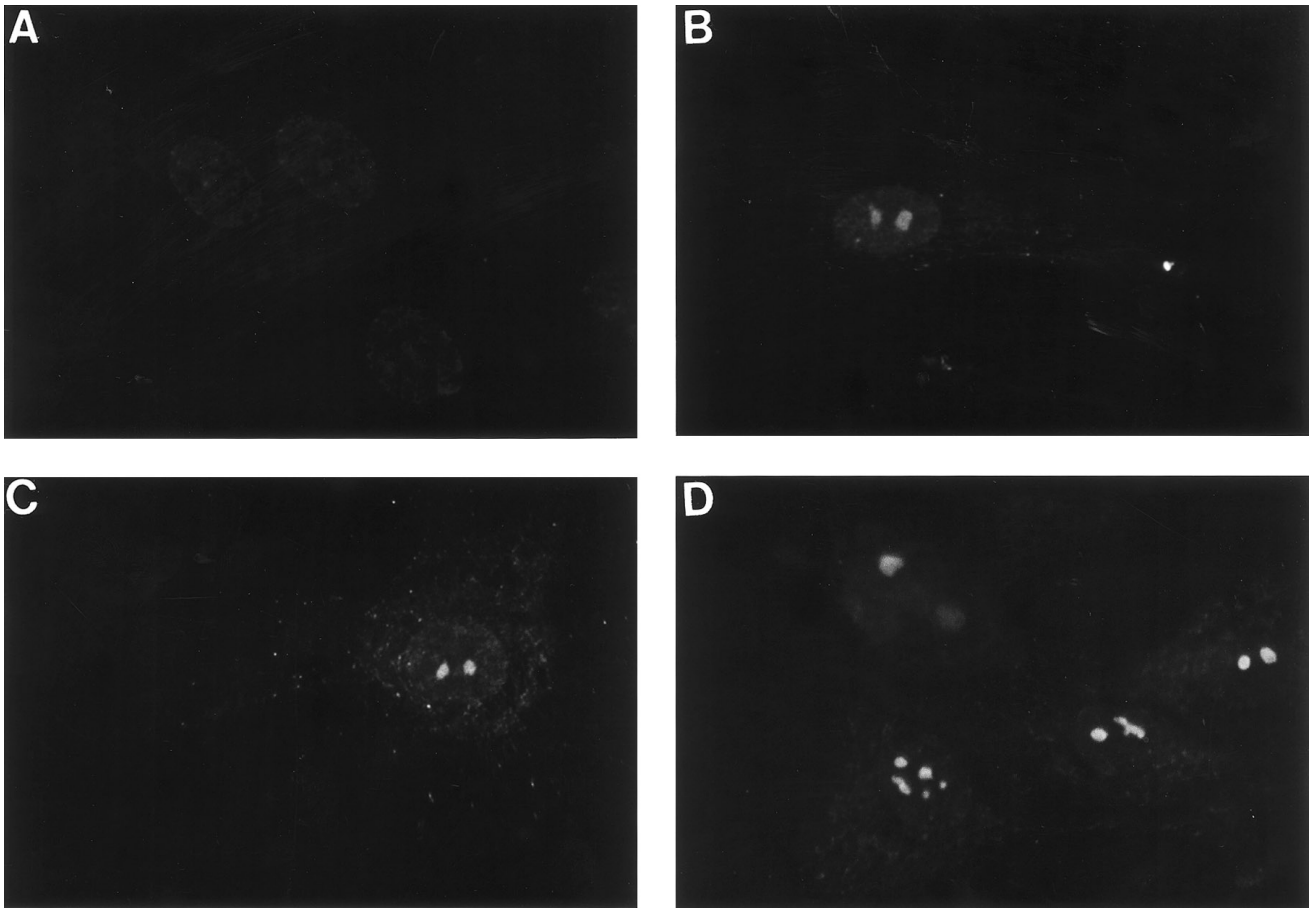

FIG. 1. Immunofluorescent detection of nuclear angiogenin in HUAE cells. Cells were incubated with 1 μg/ml of human angiogenin for 1 (A), 2 (B), 15 (C), and 120 (D) min, respectively, at 37 °C and immunostained with anti-angiogenin mAb 26-2F and FITC-labeled goat $F(ab')_2$ anti-mouse IgG. Original magnification, 100×.

ity of the drugs at the concentration used was confirmed by the fact that the microtubule network (Fig. 2A) disappeared after treatment with colchicine (Fig. 2B) and nocodazole (Fig. 2C), and formed tubulin paracrystals after treatment with taxol (Fig. 2D). Fig. 3 shows that the nucleolar accumulation of angiogenin is not affected by the disruption of microtubules. Immunofluorescent staining of angiogenin in colchicine (Fig. 3B), nocodazole (Fig. 3C) and taxol (Fig. 3D) treated cells was more or less the same as in untreated cells (Fig. 3A). These data suggest that the microtubule system is not involved in nuclear translocation of angiogenin. Moreover, treatment of cells with 2-methoxyestradiol, an inhibitor of FGF-induced angiogenesis which binds to microtubules at the colchicine site (21), did not change the nucleolar accumulation of angiogenin in HUAE cells (data not shown).

Effect of lysosomal inhibitors on nuclear translocation of angiogenin. Internalization by receptor-mediated endocytosis has been demonstrated to be a necessary early step in nuclear translocation of angiogenin (18). Since the internalized ligand-receptor complex in the endocytotic vesicles very often undergoes endosomal and lysosomal processing and degradation (27), we examined the involvement of lysosomes in nuclear translocation of angiogenin. Cells were treated at 37 °C for 16 hr with leupeptin, an inhibitor of the main lysosomal protease cathepsin B, and incubated with 1 μg/ml angiogenin in the presence of leupeptin. Immunofluorescent staining showed that nuclear translocation of angiogenin (Fig. 4A) was not affected with treatment of 25 μg/ml leupeptin (Fig. 4B), suggesting that lysosomal processing is not required for nuclear translocation. In order to know whether lysosomal targeting of angiogenin competes with nuclear translocation, the cells were treated with the lysosomotropic agent chloroquine which increases lysosomal pH by up to 2 units (23). Immunofluorescent staining showed that nuclear translocation of angiogenin was not affected after 1 hr treatment with 200 μM chloroquine (Fig. 4C). Increased cytoplasmic staining of angiogenin was observed after treatment with both leupeptin and chlo-

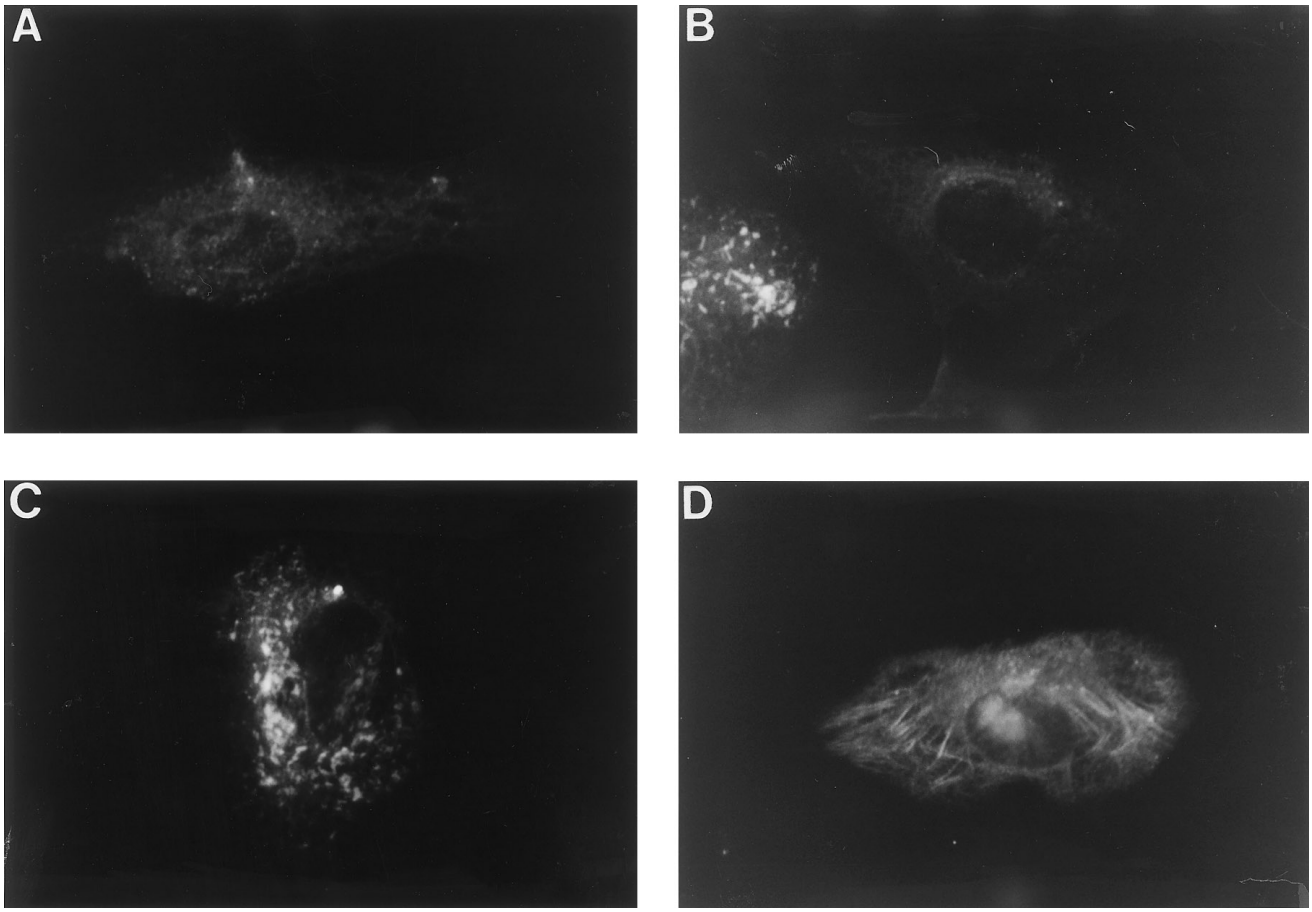

FIG. 2. Immunostaining of microtubules in HUAE cells. Cells were not treated (A) or treated with 20 μg/ml of colchicine (B), 20 μg/ml of nocodazole (C), or 50 μg/ml of taxol (D) at 37 °C for 2 hr, and stained with rabbit anti-tubulin IgG and FITC-labeled goat $F(ab')_2$ anti-rabbit IgG. Original magnification, 100×.

roquine, presumably resulting from the inhibition of lysosomal degradation. It appeared more punctate and more clustered in the perinuclear region in leupeptin treated cells (Fig. 4B), whereas more randomly distributed through the cytoplasm in chloroquine-treated cells (Fig. 4C). These data suggested that lysosomal accumulation and processing is neither required for, nor does it compete with, nuclear translocation of angiogenin. Increasing the chloroquine concentration to 400 μM resulted in a marked morphological change (round up) and reduced nucleolar staining although both nuclear and cytoplasmic angiogenin appeared to increase (data not shown).

Effect of tyrosine kinase inhibition on nuclear translocation of angiogenin. Since the receptors for most angiogenic proteins are tyrosine kinases, we examined whether tyrosine kinase activity of the cells is required for nuclear translocation of angiogenin. HUAE cells were treated with 100 μg/ml of the inhibitor genistein or daidzein (as a control) at 37 °C for 1 hr, and examined for nuclear translocation of angiogenin. Immunostaining of nuclear angiogenin in cells treated with genistein (Fig. 5B) or daidzein (Fig. 5C) showed no difference from that of untreated cells (Fig. 5A). Thus, tyrosine kinase is probably not required for nuclear translocation of angiogenin in HUAE cells.

DISCUSSION

Human angiogenin undergoes rapid nuclear translocation in HUAE cells. Translocation occurs faster in HUAE cells than in CPAE cells. It takes 15 min for angiogenin to reach saturation in the nucleolus of HUAE cells, whereas about 60 min is required for angiogenin to appear in the nucleus of CPAE cells (18). However, the internalization and translocation pathways utilized by angiogenin seem to be similar in both types of cells. As in CPAE cells, no nuclear angiogenin was detected in HUAE cells when incubation was carried out at 4 °C or when the angiogenin mutant R33A, in which the essential arginine residue of the NLS is mutated to alanine, was used. It is likely that angio-

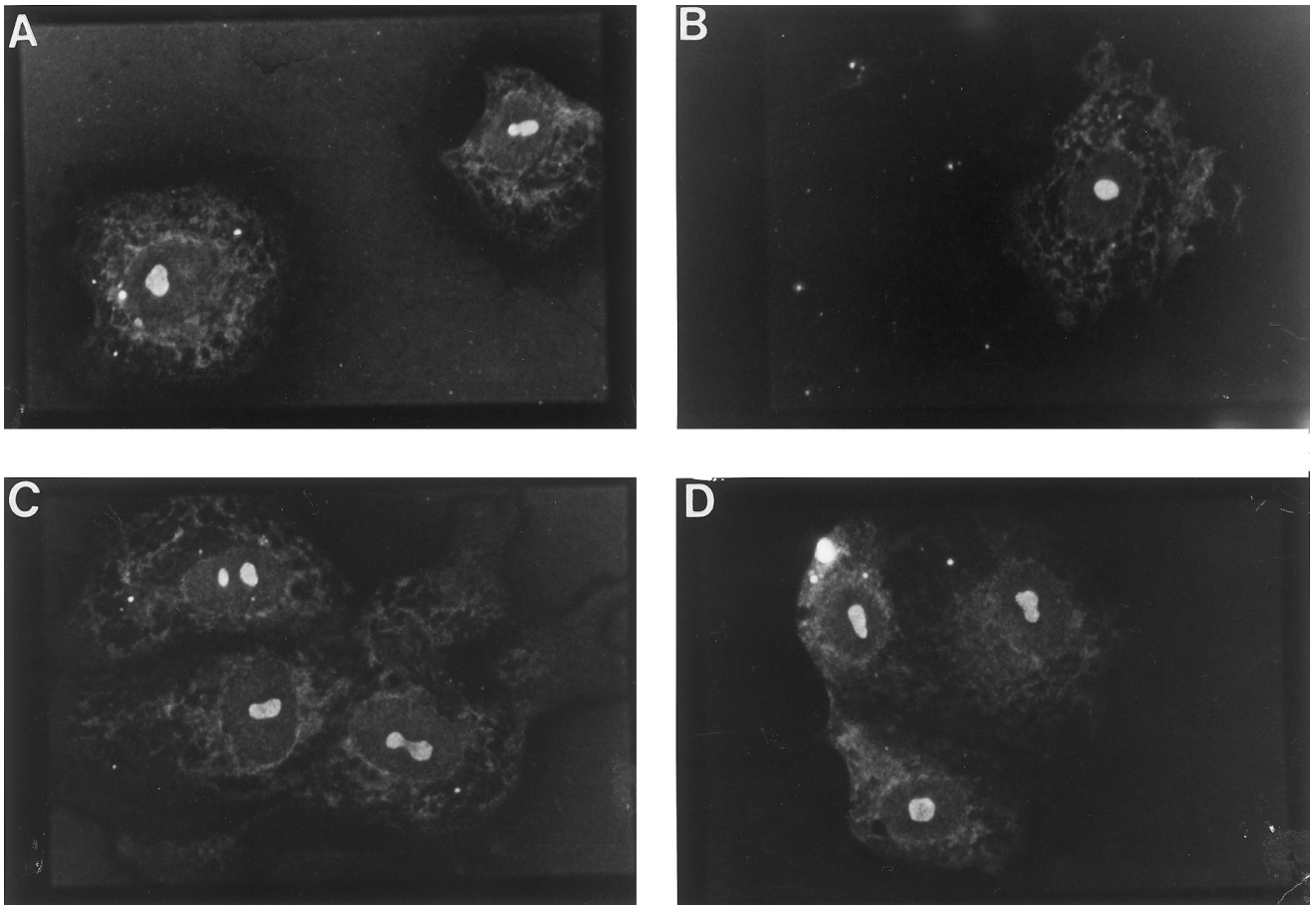

FIG. 3. Immunofluorescent detection of nuclear angiogenin in microtubule-disrupted HUAE cells. Cells were not treated (A) or treated with 20 μg/ml of colchicine (B), 20 μg/ml of nocodazole (C), or 50 μg/ml of taxol (D) at 37 °C for 2 hr, incubated with 1 μg/ml of angiogenin at 37 °C for 30 min in the presence of each individual drugs, and immunostained with anti-angiogenin mAb 26-2F and FITC-labeled goat $F(ab')_2$ anti-mouse IgG. Original magnification, 100×.

genin is internalized by receptor-mediated endocytosis and translocated to the nucleus by NLS-assisted nuclear import in HUAE cells, as has been established in CPAE cells (18).

The microtubule network is known to be required for delivery of internalized surface components to lysosomes (24-26), and for the movement of both endosomes and lysosomes and their final accumulation in clusters in the perinuclear region of the microtubule organizing center. It is also known that an intact microtubule network is required for rapid intracellular reorganization and nuclear translocation of the vitamin D receptor (27). To understand whether internalized angiogenin, which most likely is contained in vesicular compartments or endosomes, is translocated to the nucleus by a microtubule-mediated pathway, we carried out a series of experiments to examine the effects of microtubule-disrupting drugs on the nuclear translocation process of angiogenin. We observed that HUAE cells were able to correctly internalize and translocate angiogenin to the nucleolus after their microtubules were destroyed by colchicine, nocodazole, or taxol. Angiogenin was detected in the nucleolus after 2 min incubation in both colchicine-treated and untreated cells, indicating that disruption of microtubules did not inhibit or even slow down the process of nuclear translocation. Therefore, it seems that nuclear translocation of angiogenin is not mediated by microtubules, which is in line with reports that nuclear translocations of growth hormone (3), heat shock proteins 90 and 72 (28), adenovirus E1A protein (29), and the glucocorticoid (30), progesterone (31) and estrogen (32) receptors, are all mediated by microtubule-independent pathways. Nuclear translocation of growth hormone is particularly relevant to that of angiogenin. Lobie *et al.* reported that growth hormone injected intravenously into rats undergoes rapid uptake and nuclear translocation in hepatocytes (3). It was observed in the nuclei of these cells 2 min after injection and reached a maximum at 30 min. Nuclear translocation of exogenous growth hormone in

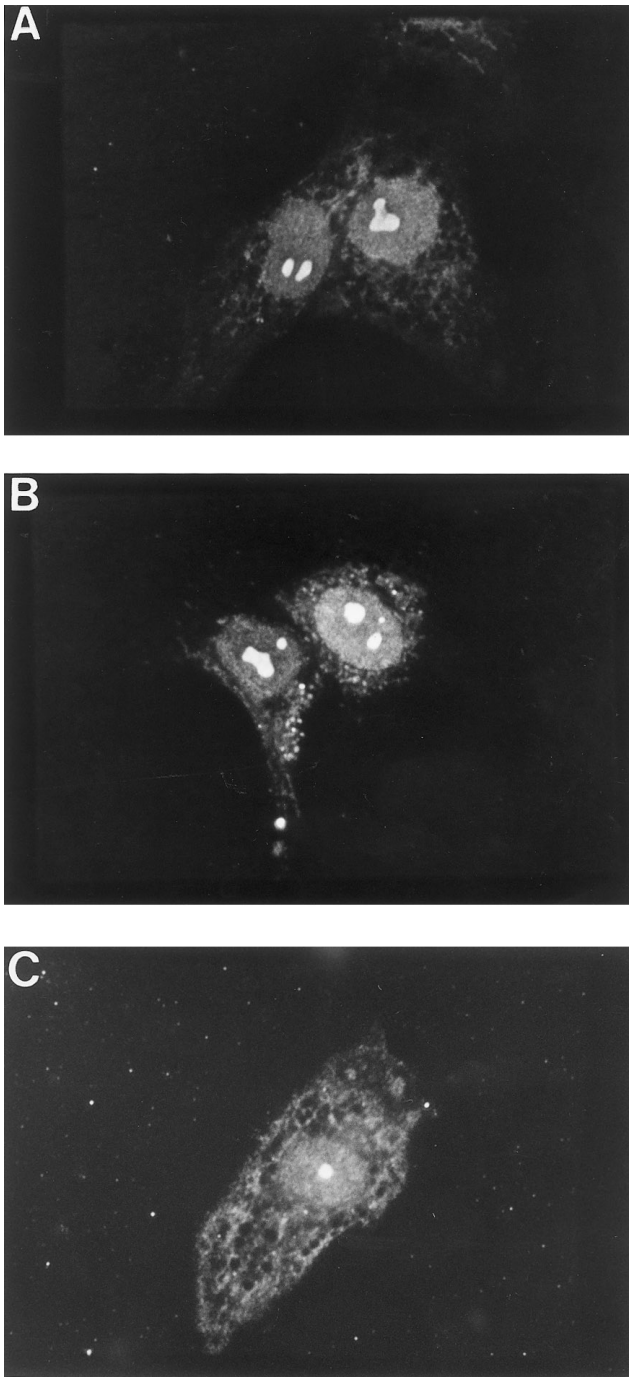

FIG. 4. Immunofluorescent detection of nuclear angiogenin in lysosomal inhibitor-treated HUAE cells. Untreated cells (A) or cells treated with 25 μg/ml of leupeptin for 16 hr (B) or with 200 μM chloroquine for 1 hr (C) were examined for nuclear translocation of angiogenin by immunostaining with mAb 26-2F and FITC-labeled goat $F(ab')_2$ anti-mouse IgG. Original magnification, 100×.

growth hormone receptor cDNA-transfected CHO4-638 cells is similarly rapid. More remarkable, no effects were observed when all three cytoskeleton systems (microtubule, microfilament and actin) were disrupted (3). Disruption of the microtubule system had no effect on nuclear translocation of angiogenin in HUAE cells, sug-

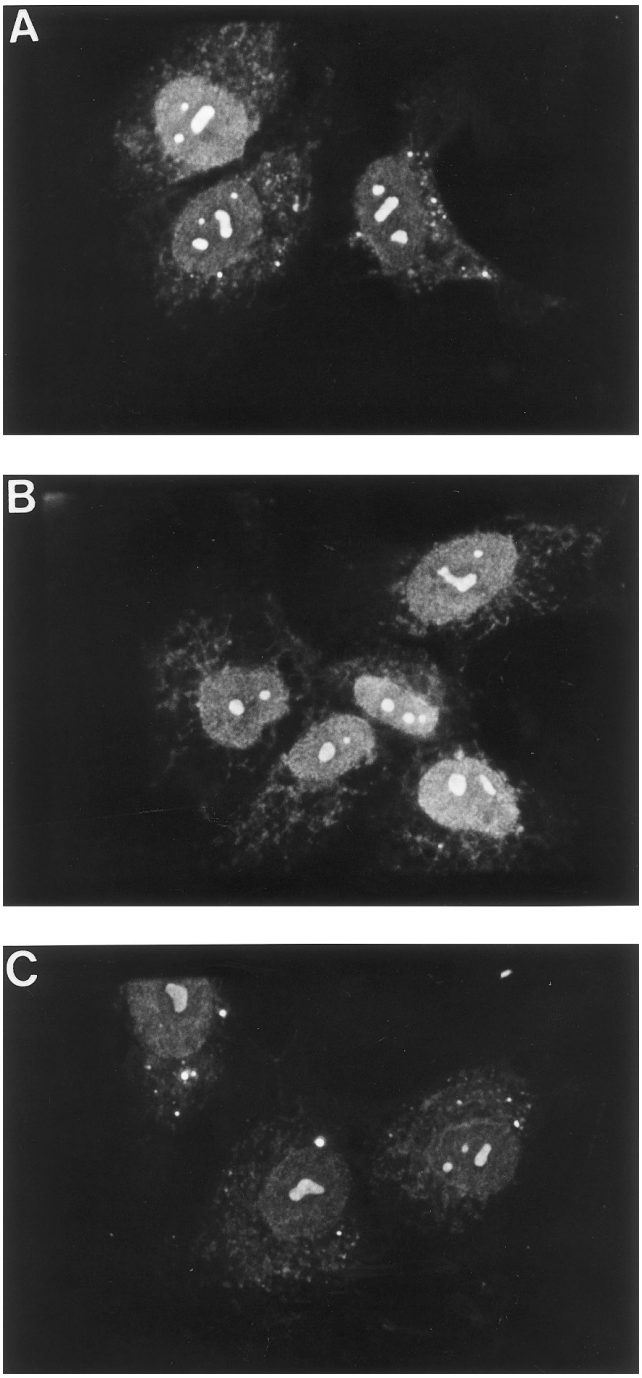

FIG. 5. Immunofluorescent detection of nuclear angiogenin in HUAE cells treated with a tyrosine kinase inhibitor. Control cells (A) or cells treated with 100 μg/ml of genistein (B) or the inactive analogue daidzein (C) were incubated with 1 μg/ml of angiogenin in the presence of the corresponding agents and immunostained by mAb 26-2F and FITC-labeled $F(ab')_2$ anti-mouse IgG. Original magnification, 100×.

gesting that the cells utilize a translocation mechanism for angiogenin that does not operate through microtubules. This is interesting considering the well known interaction between microtubules and endosomes (20) where internalized angiogenin is probably situated. On the other hand, it has been reported that only late endosomal traffic is dependent on microtubules (33), whereas early endosomal function does not require an intact microtubule network (34). It remains to be investigated whether an early endosomal process is a necessary step for nuclear translocation of angiogenin. Since internalization of angiogenin occurs by receptor-mediated endocytosis, this is certainly a favorable hypothesis. Whether or not angiogenin is released from endosomes into the cytoplasm prior to entering the nucleus also remains to be established.

Lysosomotropic agents such as chloroquine, ammonium chloride and bacitracin increase the pH of lysosomes and late endosomes (23), thereby reducing the lysosomal degradation of internalized proteins. For example, nuclear translocation of epidermal growth factor in cultured bovine corneal endothelial cells was increased 25 fold after the cells were treated with 50 μM chloroquine for 2 hr (35). Pretreatment with chloroquine or ammonium chloride enhanced nuclear translocation of growth hormone (3) and interleukin-1α (36) as well. However, nuclear translocation of angiogenin in HUAE cells was not affected after the cells were treated with 200 μM chloroquine, indicating that lysosomal targeting of angiogenin does not compete with nuclear translocation. Pretreatment of the cells with 25 μg/ml leupeptin, an inhibitor of the main lysosomal protease cathepsin B, also did not change the nuclear translocation of angiogenin, suggesting that lysosomal processing is similarly not required for nuclear translocation of angiogenin. These data do not indicate, however, that angiogenin is not taken up and processed by lysosomes altogether, only that nuclear and lysosomal translocations of angiogenin in HUAE cells do not interfere with each other. Thus the immunofluorescent staining pattern in the cytosol changes markedly in response to these treatments. There was much greater cytosolic staining after chloroquine or leupeptin treatment likely reflecting decreased degradation of angiogenin in lysosomes owing to the inhibition of the lysosomal hydrolytic activities. In leupeptin-treated cells, clusters of cytosolic angiogenin appeared in the perinuclear region, whereas in chloroquine-treated cells, cytosolic angiogenin was distributed throughout the cytosol. These phenomena may result from the different properties of leupeptin and chloroquine. Leupeptin only inhibits cathepsin B activity. It does not affect the translocation of internalized angiogenin into lysosomes. Therefore, angiogenin is taken up by lysosomes and appears as punctate staining along the perinuclear region where most of the lysosomes are located. However, chloroquine may nonspecifically raise the pH of acidic compartments which include late endosomes and lysosomes. It may then affect the accumulation of angiogenin in lysosomes. As a consequence, cytosolic angiogenin is more randomly distributed. It is interesting to observe that angiogenin undergoes lysosomal as well as nuclear translocation. Since lysosomal angiogenin was detected only when lysosomal inhibitors were present, it suggests that lysosomal degradation of angiogenin occurs very fast. The degradation of angiogenin within the lysosomal system may have physiological meaning in that it allows cells to dispose of excess angiogenin, an important consideration since cytosolic angiogenin is a potent cytotoxin (37).

Receptors for FGF (38), epidermal growth factor and nerve growth factor (39), platelet-derived growth factor (40), insulin (41), and prolactin (42) have all been reported to be nuclear associated or translocated to the nucleus. Many kinases apparently central to signal transduction are also translocated to the nucleus when activated (43, 44). Rao et al. reported that nuclear translocation of prolactin requires activation of tyrosine kinase. Thus, addition of tyrosine kinase antagonists significantly reduced nuclear translocation of prolactin (45). We have found that nuclear translocation of angiogenin in HUAE cells was not affected by the tyrosine kinase inhibitor genistein, suggesting that activation of tyrosine kinase is not required for nuclear translocation of angiogenin. Recently we identified a receptor for angiogenin on the surface of human endothelial cells (12). However, we do not yet know whether this receptor is a transmembrane tyrosine kinase. It would be of great interest to investigate whether the receptor is cotranslocated to nucleus with angiogenin as is the case with basic FGF and its receptor (38).

It is important to point out that immunofluorescence is a qualitative assay. To investigate the thermodynamics of nuclear translocation of angiogenin, the kinetics of nuclear association and dissociation, and the effect of various agents and conditions, a more quantitative assay would be necessary.

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