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Internalization and Processing of Human Angiogenin by Cultured Aortic Smooth Muscle Cells

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Running title: Internalization of angiogenin

Abbreviations: dAng, denatured angiogenin (by reduction and alkylation); SMC, aortic smooth muscle cells.

ABSTRACT

Human angiogenin is a 14-kDa plasma protein with angiogenic and ribonucleolytic activities. Angiogenin binds specifically to aortic smooth muscle cells, activates second messenger pathways, and inhibits their proliferation. Human and bovine aortic smooth muscle cells were used to study the internalization and intracellular fate of human angiogenin at 37°C. Using a specific antibody against angiogenin, we found that the internalized native protein was localized in the perinuclear region at 30 min and then dispersed throughout the cytoplasm. In conditions favoring receptor-mediated endocytosis, internalization of iodinated angiogenin showed a first peak at 5 min and then further increased for up to 24 h. The half-life of the molecule, calculated as 12 h in chase experiments, could contribute to its intracellular accumulation. In cell extracts, in addition to the 14-kDa protein, a 8.7-kDa fragment was observed at 24 h, and three fragments with molecular mass of 10.5, 8.7, and 6.1 kDa were detected at 48 h. Our data point to a specific internalization and processing of human angiogenin by aortic smooth muscle cells.

INTRODUCTION

Angiogenin is one of the most potent inducers of neovascularization in experimental models *in vivo* (1). The human 14-kDa protein shares 35% amino acid sequence identity with human pancreatic ribonuclease, and has conserved essential active-site residues (2). Angiogenin has ribonucleolytic specificity for ribosomal and transfer RNA (3). A functional enzymatic active site and a cell-binding domain are required to induce angiogenesis (4). Angiogenin binds to endothelial cells (5-7) and vascular smooth muscle cells (8), and activates second messenger pathways (9,10). Furthermore, angiogenin binds tightly to ribonuclease inhibitor, an intracellular protein (11) that abolishes both the enzymatic and angiogenic activities of angiogenin (12).

The specific interactions of angiogenin with vascular smooth muscle cells, together with its ribonucleolytic activity and the intracellular location of the ribonuclease inhibitor, led us to study the internalization and intracellular fate of angiogenin in aortic smooth muscle cells (SMC). We used radiolabeled angiogenin, native angiogenin, and a specific polyclonal antibody. The specificity of the processes studied was assessed by using denatured angiogenin (dAng), which has been shown to exhibit neither ribonucleolytic nor angiogenic activity.

MATERIALS AND METHODS

*Materials***.** Human recombinant angiogenin with the N-terminal pyroglutamyl residue (wild-type angiogenin) produced either in eucaryotic cells (13) or in *Escherichia coli* (14) was a gift from R. Shapiro (Boston, MA). It was also prepared according to Shapiro and Vallee (15).

Bovine serum albumin (BSA), fatty-acid-free BSA, insulin, transferrin, ovalbumin, trypsin inhibitor and yeast tRNA (type X) were from Sigma Chemical Co. (S^t Louis, MO). Culture media, trypsin and gentamycin were from Gibco Laboratories (Grand Island, NY) and fetal bovine serum (FBS) was from Boehringer Mannhein (Germany) and Eurobio (Toulouse, France). Sera were tested for the absence of mycoplasma and were heat-inactivated before use. Angiogenin was ^{125}I-labeled by the chloramine-T method (16) using $Na^{125}I$ (ICN Biomedicals Inc., Costa Mesa, CA), and then purified by heparin-Sepharose affinity chromatography (Pharmacia, Uppsala, Sweden) as described elsewhere (8). Specific activity was 2.3-4.6 $x10^5$ cpm/ng (1.2-3.7 Ci/μmol) corresponding to about 1 to 2 atoms of iodine per molecule. Chemicals for SDS-polyacrylamide gel electrophoresis and molecular mass markers were from BioRad (Hercules, CA), and acrylamide was from Serva (Heidelberg, Germany). All chemicals were of analytical grade.

A rabbit polyclonal antibody was prepared against angiogenin. It was purified by chromatography on a G-protein-Sepharose column and then by affinity chromatography on angiogenin immobilized in a CH-Sepharose 4B gel (Pharmacia). The specificity of the anti-

angiogenin antibody was checked by ELISA, western immunoblotting and immunodiffusion. Goat serum and goat anti-rabbit IgG were from Sigma.

Cells and media. Primary bovine SMC were a gift from P. D'Amore (Boston, MA) and were grown in Dulbecco's modified Eagle's medium (DMEM), 4.5 g/l glucose, 2.2 g/l sodium bicarbonate, 10% FBS for fewer than ten subcultures. Human SMC immortalized by the E6 and E7 open reading frames of human papillomavirus type 16 (AALTR 16-2 cells), a gift from J. K. McDougall (Seattle, WA), were propagated essentially as reported elsewhere (17), without antibiotics. AALTR 16-2 cells were used between 20 and 26 subcultures. Bovine SMC were cultured in serum-free medium in the conditions described in (18). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2, 95% air. The number of cells per culture was determined by counting trypsinized cells with a cell counter (Coultronics, France).

Immunofluorescence staining of cell monolayers. Bovine SMC (15 000-20 000 cells/cm^2) cultured for two days in DMEM/10% FBS were washed twice with phosphate-buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 6.5 mM Na_2HPO_4, 1.46 mM KH_2PO_4, pH 7.4) and then incubated in DMEM/1 mg/ml BSA with or without 100 ng/ml angiogenin for 3 h at 37°C in humidified air containing 5% CO_2. In parallel experiments, cells were incubated with angiogenin (100 ng/ml, 7 nM) in binding buffer (20 mM Mops, 130 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 3 mM KCl, 1 mg/ml BSA, pH 7.2) for 3 h at 4°C. After washing twice with PBS for 5 min, the cells were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min. After washing once with PBS, the remaining free aldehyde groups were blocked by adding 50 mM NH_4Cl for 10 min. The cells were then washed twice with PBS and permeabilized by adding 0.5% Triton X-100 in PBS for 5 min. Following three rapid washes with PBS, saturation was performed with PBS, 7% goat serum, 0.5% ovalbumin (blocking buffer) for 30 min at room temperature. Cells were then exposed to 6 μg/ml angiogenin-specific IgG in blocking buffer overnight at 4°C. After washing twice with PBS for 5 min, the cells were incubated with blocking buffer for 30 min and the bound antibody was revealed by 2 h of incubation at room temperature with TRITC-labeled goat anti-rabbit IgG diluted 1:200 in blocking buffer. Finally, the cells were washed twice with PBS and once with distilled water, then mounted in Mowiol (Calbiochem). In control experiments, the primary antibody was either omitted from the incubation medium or replaced by the IgG fraction not retained by the angiogenin-affinity chromatography column. Photographs were taken with an Olympus phase-contrast microscope with fluorescence attachment (BH2-RFCA).

Reductive alkylation of angiogenin. Reductive alkylation of native and iodinated angiogenin was performed according to Anfinsen and Haber (19). Two micrograms (52 nM) of angiogenin diluted in 20 mM Mops, 130 mM NaCl, 0.2 mg/ml BSA pH 7.2 was stirred in 8

M urea, 0.28 M ß-mercaptoethanol, pH 8.5 in a total volume of 2.8 ml, for 4 h at room temperature under nitrogen. The reaction mixture was desalted by filtration on a PD10 Sephadex G25-M column (Pharmacia) equilibrated in 0.1 M acetic acid, and pH was adjusted to 7.8 by adding 0.6 vol. of 1 M Hepes. Alkylation was then performed in the presence of 25 mM iodoacetamide for 45 min at 27°C in the dark. After desalting the sample by gel filtration on G25 in 20 mM Mops, 130 mM NaCl, 0.2 mg/ml BSA pH 7.2, the denatured angiogenin (dAng) was concentrated by ultrafiltration through Centricon centrifugal concentrators (10 000 MW cut-off, Amicon, Beverly, MA) at 5 000 x g for 1 h at 4°C.

Ribonucleolytic assay. The ribonucleolytic activity of native and alkylated angiogenin was assessed in an agarose gel-based assay (20). The reaction mixture, containing 8 mM Tris pH 7.5, 12 mM NaCl, 1 μg of yeast tRNA and 10, 25, 50 or 100 ng of angiogenin in a volume of 10 μl, was incubated for 1 or 2 h at 37°C. The reaction was stopped by chilling on ice. Enzymatic degradation was analyzed immediately, by electrophoresis of the samples in 1.5% agarose gel containing 0.5 μg/ml ethidium bromide.

Cellular uptake of ^{125}I-angiogenin. Cells were seeded at a density of 15 000-20 000/cm^2 in 4- or 6-well plates (Falcon Becton Dickinson, NJ) and cultured for 2 days before experiments. Cells were washed three times with PBS at 37°C and incubated with native or denatured ^{125}I-angiogenin (0.2 nM), for the times indicated at 37°C in humidified air containing 5% CO_2. Alternatively, cultures were pre-incubated in DMEM/1 mg/ml BSA for 3 h at 37°C before adding angiogenin, to dissociate any secreted angiogenin or interacting molecules from serum bound to the cell surface. The following buffers were used as indicated: DMEM/10% FBS, DMEM/1 mg/ml BSA, DMEM/1 mg/ml fatty-acid-free BSA/10 μg/ml transferrin/30 μg/ml insulin/50 μg/ml gentamycin, binding buffer. At the times indicated, cells were cooled to 4°C and all subsequent operations were done at this temperature. After washing once with cold PBS, cell monolayers were incubated for 2 min in 1 M NaCl, 0.2 M CH_3COOH pH 2.5 (21) to release surface-bound angiogenin. They were then washed once with PBS, dissociated by trypsin treatment and collected by centrifugation at 450 x g for 5 min at 4°C in the presence of 1 mg/ml trypsin inhibitor per mg trypsin. Alternatively, to release at once bound ^{125}I-angiogenin, the cell monolayer was washed four times with cold PBS and then treated with trypsin, without the acid treatment. The two protocols gave similar results. The pellets were solubilized in 50 μl of 2x electrophoresis sample buffer (1x: 50 mM Tris-HCl, 5% glycerol, 1% SDS, 0.14 M β-mercaptoethanol, 0.001% bromophenol blue, pH 6.8). Radioactivity was counted using a LKB model 1275 Minigamma counter at 80% efficiency. In control experiments, cells were cooled to 4°C, washed four times with cold PBS and then incubated with ^{125}I-angiogenin for 10 min. They were then treated as above.

Fate of internalized ^{125}I-angiogenin. To study possible degradation of angiogenin, cell pellets were analyzed by SDS-polyacrylamide gel electrophoresis on a 3% polyacrylamide stacking gel and a 15% polyacrylamide resolving gel. To visualize ^{125}I-angiogenin and its degradation products, the dried gels were exposed to X-OMat AR autoradiography films (Kodak) at -80°C using intensifying screens.

Chase. The half-life of angiogenin was determined in 2-day cultured bovine SMC and human AALTR-16 cells plated at 15 000-20 000 cells/cm^2. Cells were incubated in the presence of 0.2 nM ^{125}I-angiogenin for 3 h in either DMEM/1 mg/ml BSA/30 μg/ml insulin/10 μg/ml transferrin or DMEM/10% FBS, at 37°C in humidified air containing 5% CO_2, then washed three times with prewarmed PBS at 37°C and finally incubated with 0.2 nM unlabeled angiogenin and 0.2 nM ^{125}I-angiogenin, respectively, for 3, 21, 45 and 69 h in culture medium at 37°C. At the indicated times, cells were placed at 4°C and treated as described above.

RESULTS

Immunofluorescence detection of internalized angiogenin. Angiogenin uptake by bovine SMC was visualized after immunocytochemical staining with a specific polyclonal antibody against human angiogenin. At 30 min and 1 h, angiogenin was located in the perinuclear area (Fig. 1B, 1D). After 3 h, bright staining was observed only in the cytoplasm (Fig. 1F). Not all the cells were labeled (Fig. 1D). No labeling was detected using either the IgG fraction not retained by immobilized angiogenin (non specific IgG; not shown) or the 2nd labeled antibody alone (Fig. 1H). Control cells (not exposed to angiogenin) were also negative, as the anti-human angiogenin IgG did not react with bovine angiogenin (not shown).

Time-course of angiogenin binding and internalization at 37°C. To examine receptor-mediated endocytosis, angiogenin internalization was studied in conditions that favored high-affinity binding, as previously determined (8). Sparse cells were incubated in binding buffer with 0.2 nM ^{125}I-angiogenin at 37°C for the times indicated. Two waves of internalization were observed (Fig. 2). Angiogenin was rapidly internalized with a maximum at 5 min followed by a second increase after 10 min. However, surface-bound angiogenin reached its maximum within 15 min and then remained constant (Fig. 2, inset). To study the possible influence of serum components such as endogenous angiogenin, bovine SMC were cultured for at least three passages in serum-free medium; similar results were obtained (not shown).

Specificity of internalization. Denatured angiogenin was used to control the specificity of angiogenin internalization, as the high isoelectric point of angiogenin (pI > 9.5) could result in extensive binding to negatively charged molecules. Reduced and alkylated angiogenin has neither ribonucleolytic nor angiogenic activity (22). Denatured angiogenin did not hydrolyze yeast tRNA (Fig. 3A). Saturation experiments were performed with the labeled alkylated molecule at 4°C and 2-day cultured bovine SMC as described elsewhere (8). Analysis of the

binding data using the LIGAND program (23) suggested a total or partial loss of recognition of high-affinity binding sites. Furthermore, competition experiments of ^{125}I-dAng binding with increasing concentrations of native angiogenin (as well as cross-linking experiments) showed that angiogenin did not affect ^{125}I-dAng binding to bovine SMC (not shown and Fig. 3B). It is worth noting that the acid wash, known to disrupt ligand-receptor interactions (8,21), had little effect on the removal of dAng from the cell surface compared to native angiogenin. Most cell-bound dAng was sensitive to trypsin treatment.

To study angiogenin internalization, 0.2 nM ^{125}I-angiogenin (native or denatured) was added to 2-day cultured bovine SMC for 3 h at 37°C in the presence or absence of serum. The results showed that the two molecules were internalized by SMC but the amounts of internalized dAng were always lower, despite its higher total binding to the cell surface. Maximal internalization of the two molecules was obtained in serum-free medium (Fig. 3C).

Internalization of angiogenin and dAng did not occur *via* the same pathway, as dAng failed to chase internalized ^{125}I-angiogenin during 48 h of incubation, whereas angiogenin chased 85% of ^{125}I-angiogenin (not shown).

Long-term internalization. The kinetics of angiogenin and dAng internalization was studied at 37°C to investigate the intracellular fate of the molecules. Two-day cultured bovine SMC were placed in serum-free medium and incubated with 0.2 nM ^{125}I-angiogenin for 15 min and 1, 3, 6 and 24 h at 37°C. The amount of internalized angiogenin increased with the incubation time (Fig. 4A), and intracellular accumulation of angiogenin was observed.

To visualize the intracellular fate of angiogenin, the cell pellets were analyzed by SDS-PAGE and autoradiography. Figure 4B shows the partial degradation of the two angiogenin forms. A single degradation product of 8.7 kDa was detected at 6 h with native ^{125}I-angiogenin. In contrast, ^{125}I-dAng was processed into fragments detected at 1 h of incubation, and its degradation was more extensive, as two bands with molecular mass of 10.2 and 8.7 kDa were visualized at 24 h.

Chase studies. Human immortalized SMC (AALTR 16-2) were used to study species specificity. Cells were incubated for 3 h with ^{125}I-angiogenin at 37°C and then washed. Internalized ^{125}I-angiogenin was chased by the same amounts of native angiogenin for 3, 21 and 45 h. The amount of internalized radioactivity fell with time and reached a plateau at 24 h. (Fig. 5A). The half-life of the molecule was calculated to be 12 h. The radioactivity released in the incubation medium supported the chase of the internalized molecule. These results were confirmed by SDS-PAGE of the cells. After a 45-h chase with unlabeled angiogenin, no ^{125}I-angiogenin was detected inside the cells. However, chase experiments with ^{125}I-angiogenin indicated that angiogenin continued to enter the cells, as intracellular accumulation of radioactivity was observed, and three degradation fragments (10.5, 8.7, and 6.1 kDa) were visualized (Fig. 5B). This processing did not occur through a lysosomal pathway, as methylamine (30 mM) and ammonium chloride (20 mM), both of which

indirectly inactivate lysosomal hydrolases, had no effect on angiogenin degradation (data not shown).

DISCUSSION

Sparse SMC express two classes of angiogenin binding site. On bovine SMC, high-affinity binding sites with an apparent dissociation constant (K_d) of 2 x 10^{-10} M bind 1 x 10^4 molecules/cell, and low-affinity binding sites (K_d = 1 x 10^{-7} M) bind millions of molecules. The number of high-affinity binding sites decreases as cell density increases, and they are no longer detected at confluence (8). They are probably cell-surface receptors, as angiogenin in the concentration range of its high-affinity K_d activates second messenger pathways (10), and inhibits SMC proliferation (8). To learn more of the intracellular function and/or regulation of angiogenin, its binding, internalization and processing by bovine and human SMC were studied at 37°C.

In conditions targeting high-affinity binding sites, ^{125}I-angiogenin binding to the cell surface reached a plateau by 15 min, which reflected a steady state among several concomitant processes, including cell-surface binding, intracellular transit and processing, and discharge of angiogenin and/or its fragments into the extracellular medium. In contrast with the rapid binding, internalization peaked at 5 min, and ^{125}I-angiogenin continued to accumulate in the cells after 15 min, for up to 24 h. This intracellular accumulation suggests that the cell surface is replenished with angiogenin receptors either recycled after internalization or supplied by a large intracellular pool. The 12-h half-life of the molecule, calculated from chase experiments, could also contribute to its intracellular accumulation. In these conditions, 5-10% of ^{125}I-angiogenin bound to the cell surface was internalized by SMC (corresponding to 400-1000 molecules/cell). The small number of internalized molecules might reflect an intracellular function.

Surface-binding and internalization of ^{125}I-angiogenin by SMC were also examined as a function of the angiogenin concentration from 0.2 to 65 nM (not shown). When the angiogenin concentration was higher than the dissociation constant for high-affinity binding sites, binding of the molecule to the cell surface reached the maximal level more rapidly, and an increase in the amount of internalized ^{125}I-angiogenin was observed. This suggests that, in addition to receptor-mediated internalization, angiogenin endocytosis occurs via other routes which could involve low-affinity binding sites.

Immunostaining experiments using an angiogenin concentration in the low-affinity K_d range (100 ng/ml) showed a perinuclear localization of internalized angiogenin at 30 min and 1 h. Similar observations have been made with endothelial cells and 1 μg/ml angiogenin, with a nucleolar location (24). However, angiogenin was dispersed in the SMC cytoplasm after 3 h of internalization.

Interestingly, internalized angiogenin is resistant to cellular degradation. In addition to the 14-kDa protein, a single 8.7-kDa fragment was observed at 24 h, and three fragments (10.5, 8.7, and 6.1 kDa) were detected at 48 h. Angiogenin processing does not occur in lysosomes, as it was not inhibited by the lysosomotropic bases ammonium chloride and methylamine (not shown) which inhibit degradative agents by raising the lysosomal pH (25). This supports the specificity of angiogenin internalization, as degradation of RNase A (68% homologous to angiogenin) occurs exclusively in lysosomes (26-28). The specificity of processing is also suggested by the rapid degradation of denatured angiogenin. Angiogenin is resistant to a wide variety of proteolytic enzymes (29) but is cleaved by human neutrophil elastase (30). Note that angiogenin belongs to the RNase family whose members are resistant to intracellular degradation (31). The small size and the basic isoelectric point could explain the long intracellular life of these molecules (28). Restricted endosomal proteolysis is used both for inactivation (e.g. insulin, glucagon and epidermal growth factor) and activation (e.g. lysosomal hydrolases, toxins and parathyroid hormone) of internalized proteins through a receptor-mediated pathway (32,33). Further studies are needed to determine whether this process is involved in the regulation of angiogenin functions.

As angiogenin is present in plasma (34), the specific binding of human angiogenin to SMC, together with its internalization and metabolism, supports the involvement of angiogenin in vessel wall homeostasis.

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Figure legends

FIG. 1. Immunolocalization of internalized angiogenin in bovine SMC. Cells were incubated with 100 ng/ml angiogenin for 0.5 h (A, B, G, H), 1 h (C, D) and 3 h (E-F) at 37°C, fixed in 4% paraformaldehyde and permeabilized, and then incubated with 6 μg/ml anti-angiogenin IgG overnight at 4°C. Bound antibody was revealed by 2-h incubation with TRITC-labeled goat anti-rabbit. **(G, H)** Control experiment without anti-angiogenin IgG. **(A, C, E, G)** Phase-contrast. Bar: 20 μm.

FIG. 2. Time-course of ^{125}I-angiogenin internalization (Ang) by bovine SMC at 37°C. Two-day cultured SMC were incubated with 0.2 nM ^{125}I-angiogenin in binding buffer. At the times indicated the monolayers were washed four times with cold PBS and the cells were collected by trypsin treatment and centrifugation. Surface-bound ^{125}I-angiogenin was released into the supernatant (inset), and internalized radioactivity was determined in the pellets. Results are means ± SD of triplicate determinations. The profiles are representative of three independent experiments.

FIG. 3. Characteristics of denatured angiogenin. (**A**) Ribonucleolytic activity on yeast tRNA. Various amounts of native and denatured angiogenin (Ang and dAng, respectively) were incubated with 1 μg of tRNA in 8 mM Tris, pH 7.5, 12 mM NaCl for 1.5 h at 37°C. The reaction products were cooled to 4°C and analyzed by electrophoresis in a 1.5% agarose gel. 1: 10 ng; 2: 25 ng; 3: 50 ng; 4: 100 ng. Cltr: incubation of 1 μg tRNA in the absence of angiogenin. (**B**) Cross-competition of ^{125}I-angiogenin and ^{125}I-dAng binding to sparse SMC by a 100-fold excess of angiogenin and dAng, respectively. SMC were incubated for 3 h at 4°C with 0.8 nM ^{125}I-angiogenin and ^{125}I-dAng in binding buffer (*: ^{125}I-angiogenin was 0.14 nM). Cell-associated radioactivity was counted in triplicate wells. Results are expressed as % of radioactivity competed in the presence of competitor. (**C**) Specificity of angiogenin internalization by SMC. Two-day sparse bovine SMC (19 000/cm^2) were incubated either in DMEM/10% FCS (serum) or in DMEM/1 mg/ml BSA/ 10 μg/ml transferrin/30 μg/ml insulin (serum-free) in the presence of ^{125}I-angiogenin and ^{125}I-dAng (0.2 nM) for 3 h at 37°C. They were then placed on ice and washed with cold PBS. An acid wash with 1 M NaCl, 0.2 M CH_3COOH, pH 2.5 was then performed and the cells were collected by trypsin treatment and centrifugation. Surface-bound radioactivity was the sum of acid-wash and supernatant counts. Internalized radioactivity was determined in the pellets. Results are means ± SD of triplicate determinations.

FIG. 4. Time-course of radiolabeled-angiogenin internalization and degradation at 37°C. (**A**) bovine SMC (20 000/cm^2) were incubated with 0.2 nM ^{125}I-angiogenin and ^{125}I-dAng, respectively, in DMEM/1 mg/ml BSA/10 μg/ml transferrin/30 μg/ml insulin for the times indicated. Then they were cooled to 4°C, washed and acid-treated for 2 min to release surface-bound angiogenin. They were then washed again, treated with trypsin and centrifuged. Supernatants were used to measure surface-bound angiogenin. Internalized radioactivity was determined in the pellets. Results are means ± SD of triplicate determinations. (**B**) Cells were analyzed by SDS-PAGE and autoradiography. The dried gels were exposed for 4 days. Bands are shown with their approximate molecular mass (kDa). Degradation of internalized ^{125}I-angiogenin (a) and ^{125}I-dAng (b). Ctlr: In control experiments, radiolabeled angiogenin was added for 10 min at 4°C, and the cells were then treated as above.

FIG. 5. Pulse-chase studies of internalized ^{125}I-angiogenin in human SMC. (**A**) AALTR 16-2 cells (18 000/cm^2) were pulsed with ^{125}I-angiogenin (0.2 nM) for 3 h and chased with 0.2 nM angiogenin (□) or ^{125}I-angiogenin (○) for 3, 21 and 45 h in DMEM/10% FCS. Then, cells were placed on ice and treated as indicated in Fig. 4. (**B**) Cells were analyzed by SDS-PAGE and autoradiography of the gel at 48h. Ctlr: In control experiments, ^{125}I-angiogenin was added for 10 min at 4°C, and the cells were then treated as above. a: Chase by ^{125}I-angiogenin; b: chase by angiogenin; Ang: ^{125}I-Ang (4000 cpm).

Figure 1

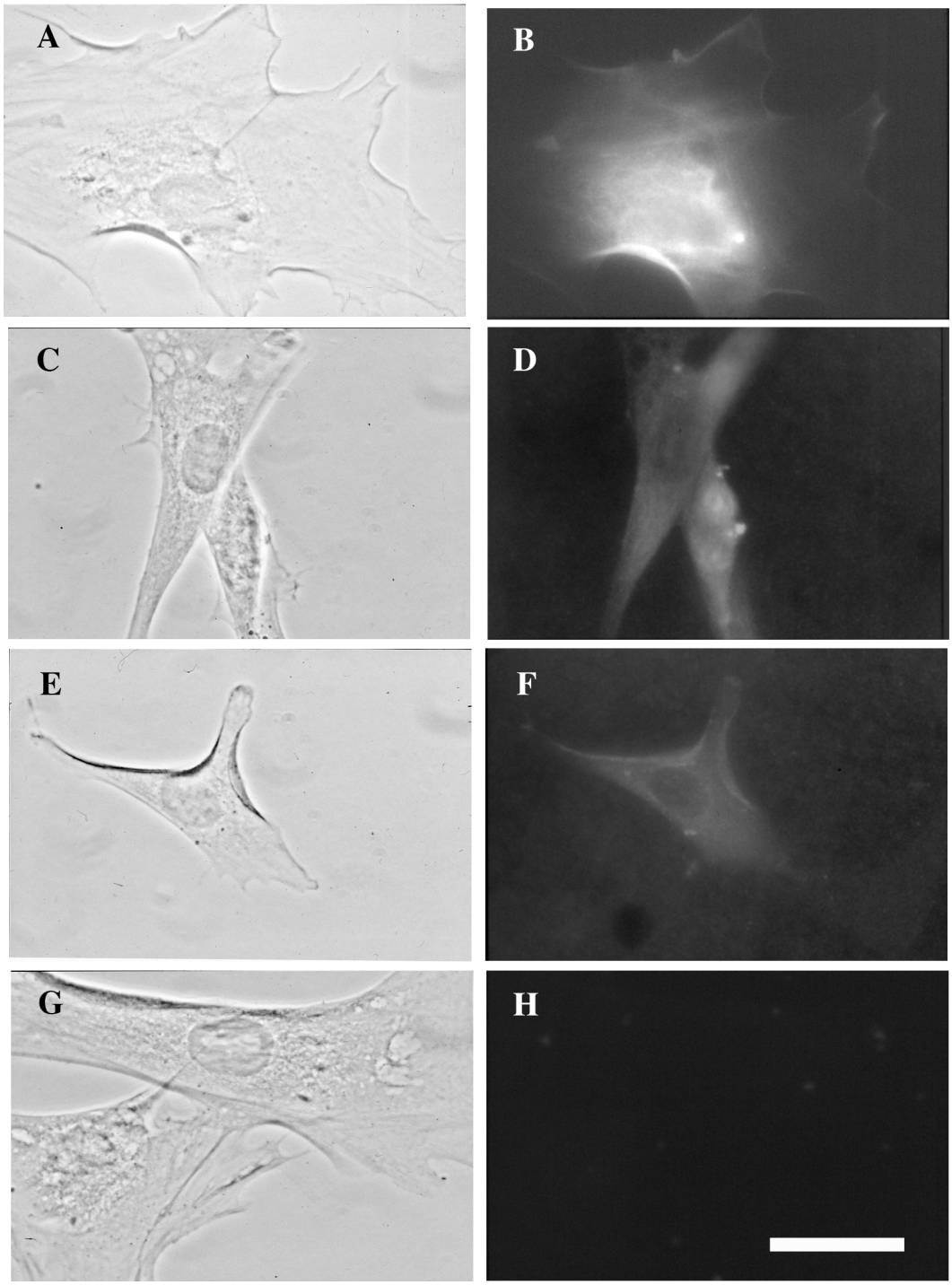


Figure 1

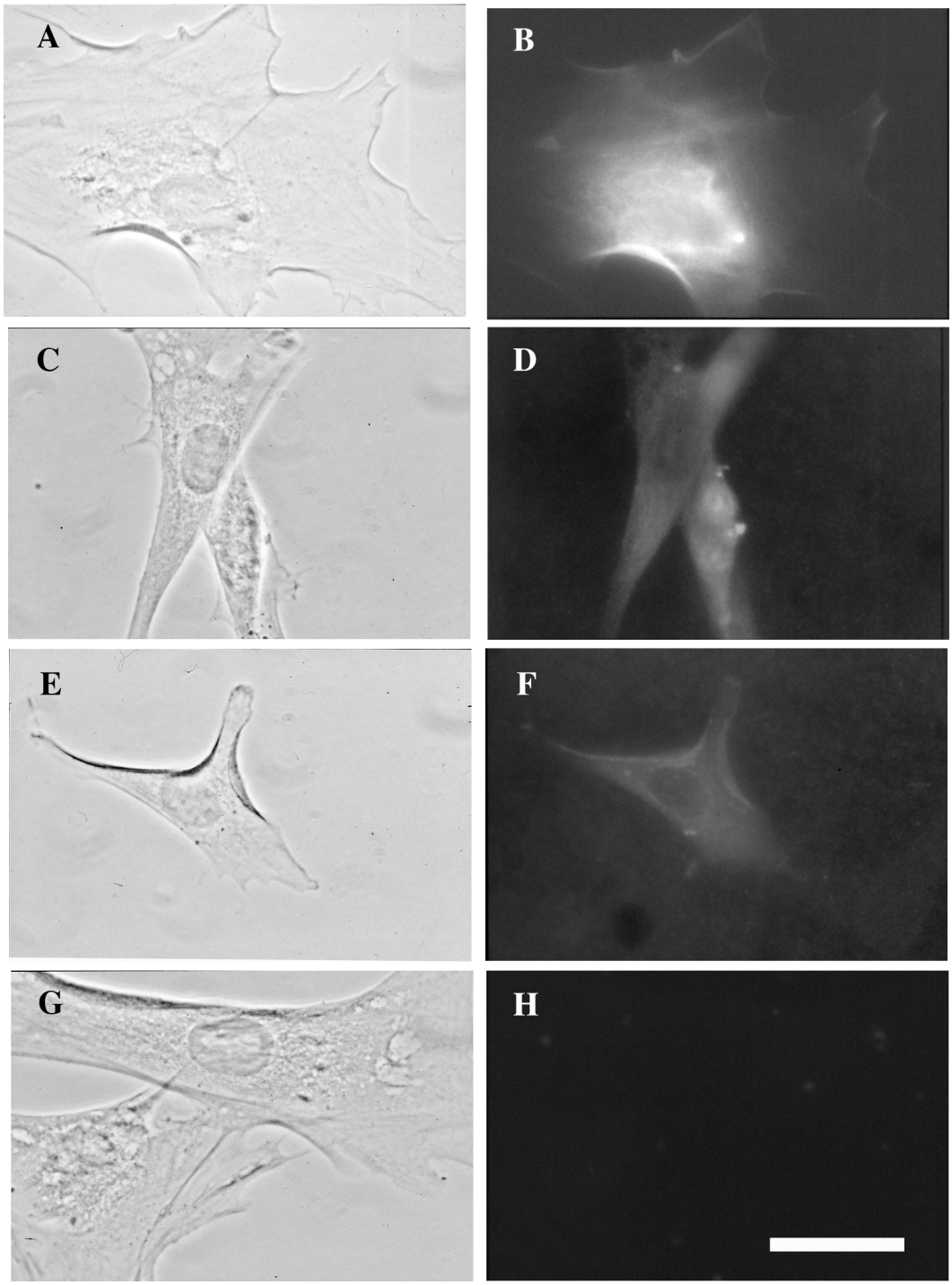


Figure 2

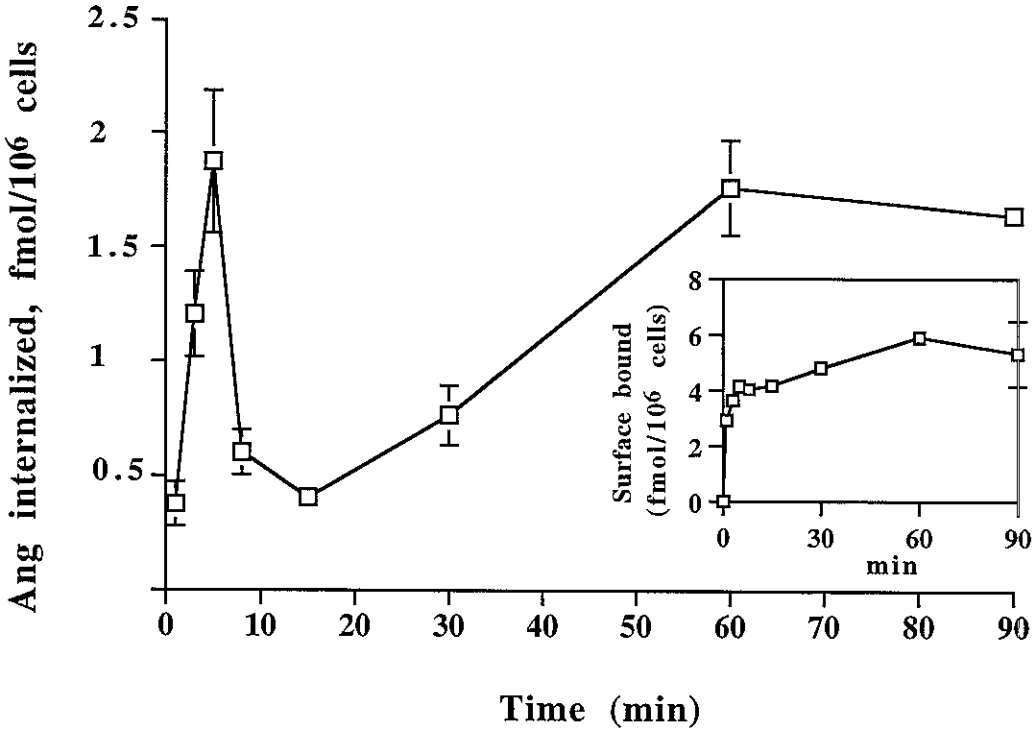
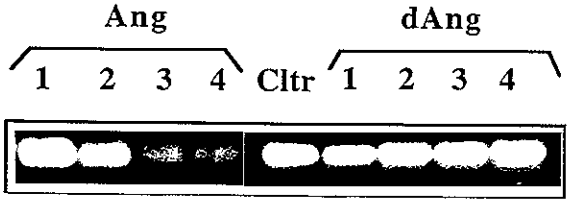

Ang internalized, fmol/10⁶ cells
2.5
2
1.5
1
0.5
0
10
20
30
40
50
60
70
80
90
Time (min)
Surface bound (fmol/10⁶ cells)
8
6
4
2
0
0
30
60
90
min

Figure 3

A

Ribonucleolytic activity of angiogenin



B

Competition of binding to SMC (%)

	unlabeled competitor	
	Ang	**dAng**
^{125}I-Ang	77 ± 10	18 ± 10 *
^{125}I-dAng	6 ± 2	38 ± 3

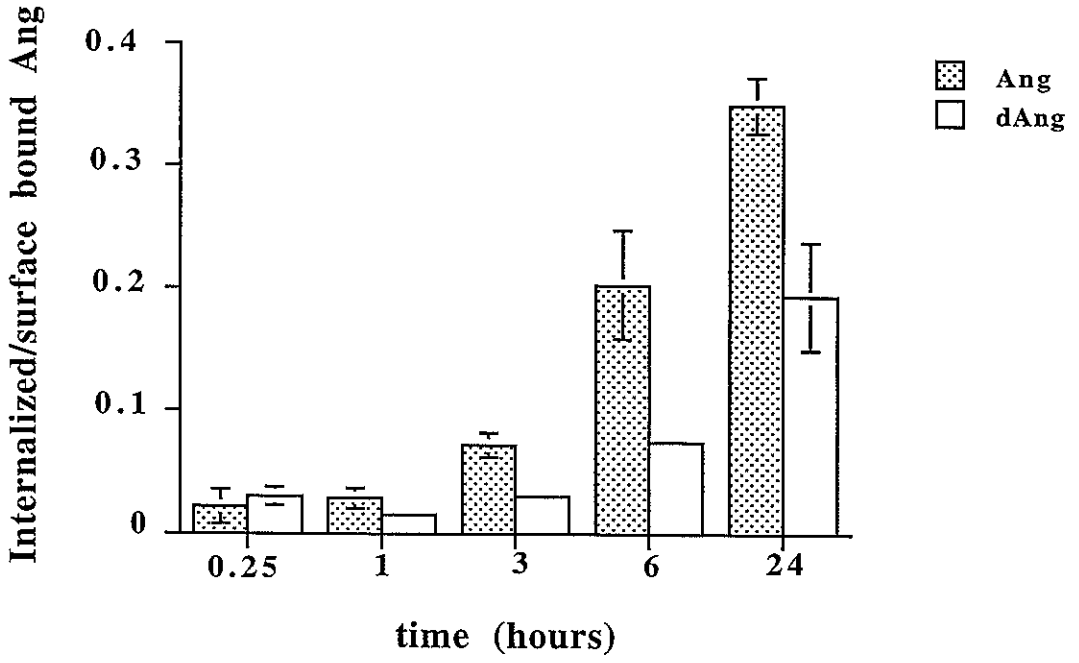
C

Internalization of Ang and dAng

	internalized (fmol/10^6 cells)		**internalized/surface**	
Experimental conditions	**^{125}I-Ang**	**^{125}I-dAng**	**^{125}I-Ang**	**^{125}I-dAng**
serum	0.83 ± 0.15	0.29 ± 0.04	0.09	0.03
serum-free	1.38 ± 0.13	0.74 ± 0.19	0.09	0.03

Figure 4

A


Internalized/surface bound Ang
0.4
0.3
0.2
0.1
0
0.25
1
3
6
24
time (hours)
Ang
dAng

B

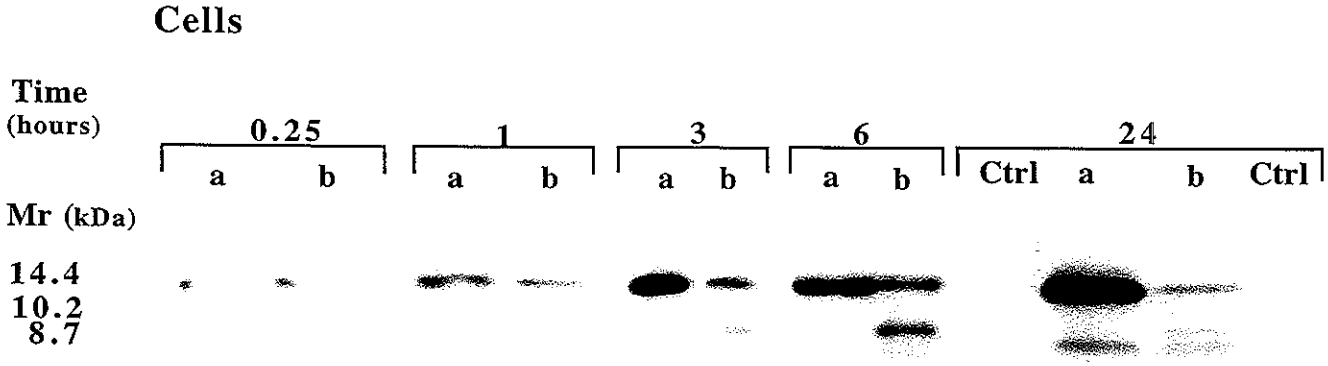
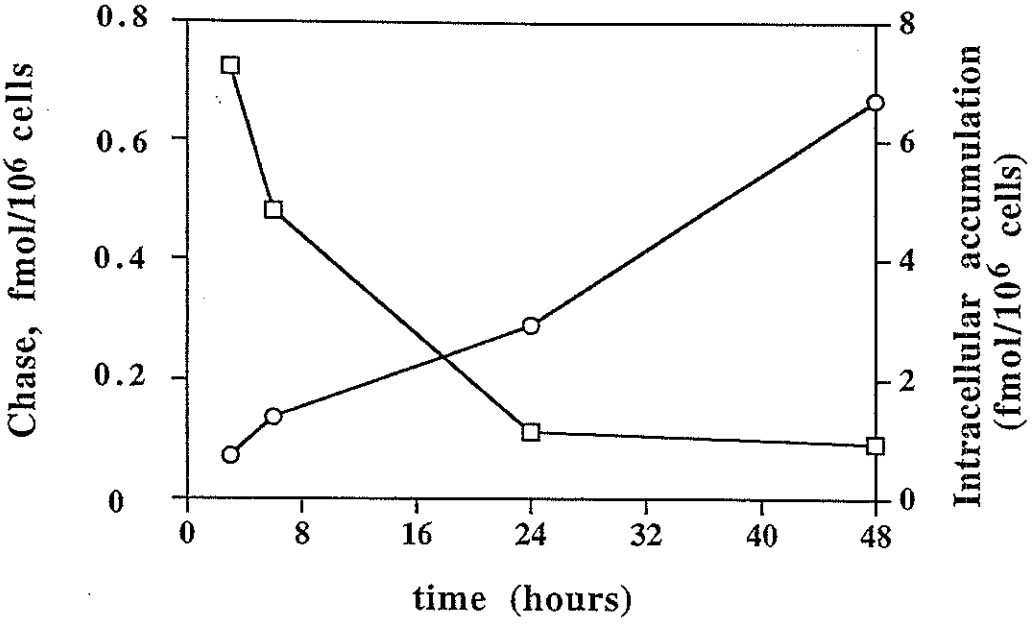
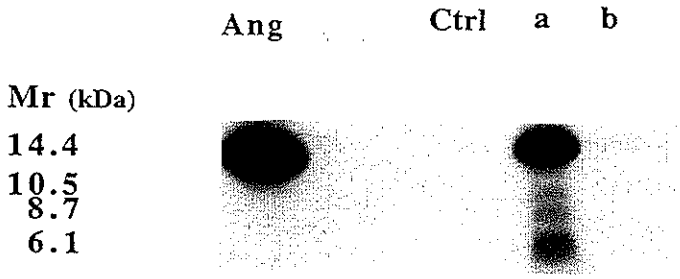

Cells
Time
(hours)
0.25
1
3
6
24
a
b
a
b
a
b
a
b
Ctrl
a
b
Ctrl
Mr (kDa)
14.4
10.2
8.7

Figure 5

A


0.8
0.6
0.4
0.2
0
Chase, fmol/10⁶ cells
8
6
4
2
0
Intracellular accumulation (fmol/10⁶ cells)
0
8
16
24
32
40
48
time (hours)

B


Ang
Ctrl
a
b
Mr (kDa)
14.4
10.5
8.7
6.1