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Actin is a binding protein for angiogenin

(angiogenesis)

GUO-FU HU, DANIEL J. STRYDOM, JAMES W. FETT, JAMES F. RIORDAN, AND BERT L. VALLEE

Center for Biochemical and Biophysical Science and Medicine, Harvard Medical School, 250 Longwood Avenue, Boston, MA 02115

Contributed by Bert L. Vallee, November 9, 1992

ABSTRACT **The 42-kDa angiogenin binding protein isolated previously has been purified to electrophoretic homogeneity. It has been identified as a member of the actin family by peptide mapping and partial amino acid sequencing. The interaction of bovine muscle actin with angiogenin is similar to that of the angiogenin binding protein. Angiogenin induces the polymerization of actin below the critical concentration for spontaneous polymerization. The interaction occurs both in solution and on a poly(vinylidene difluoride) membrane. It is inhibited by excess unlabeled angiogenin and also by platelet factor 4 and protamine, which are known inhibitors of angiogenesis. Two other angiogenic molecules, basic fibroblast growth factor and tumor necrosis factor α, bind to ^{125}I-labeled actin and can be crosslinked by a water-soluble carbodiimide. Both actin and an anti-actin antibody inhibit the angiogenic activity of angiogenin in the chicken embryo chorioallantoic membrane assay. The results indicate that the angiogenin binding protein is a cell surface actin and suggest that the reaction between angiogenin and this actin is an essential step in the angiogenesis process induced by angiogenin.**

Angiogenin is a 14-kDa protein purified initially from serum-free supernatants of an established human adenocarcinoma cell line, HT-29 (1). It was the first human tumor-derived angiogenic protein to be isolated based on its *in vivo* activity. It stimulates endothelial cells to produce diacylglycerol (2) and secrete prostacyclin (3) by phospholipase activation. It supports endothelial and fibroblast cell adhesion (4) and modulates a mitogenic effect in certain cells (5). An angiogenin binding protein (AngBP), which has properties consistent with its being a component of a cellular receptor for angiogenin, has been identified and isolated from a transformed endothelial cell line, GM7373 (6). It is a cell-surface protein with an apparent molecular mass of 42 kDa and is released from endothelial cells by exposure to heparin, heparan sulfate, or angiogenin itself.

We report here the further purification and characterization of AngBP. Tryptic peptide mapping and amino acid sequence analysis indicate that AngBP is a member of the actin family. The binding of angiogenin to bovine muscle actin and the inhibitory effect of actin and anti-actin antibodies on angiogenin-induced neovascularization on the chicken embryo chorioallantoic membrane (CAM) are also reported. The mechanism by which AngBP is released from the cell surface and the physiological significance of its presence and displacement remain to be elucidated.

MATERIALS AND METHODS

Materials. Bovine angiogenin, purified by the method of Bond and Vallee (7), was provided by R. Shapiro. ^{125}I-labeled angiogenin and ^{125}I-labeled actin of specific activities 25 μCi/μg and 10 μCi/μg, respectively (1 Ci = 37 GBq), were prepared with Enzymobeads (Bio-Rad). CNBr-activated Sepharose 6MB was from Pharmacia; bovine (A3653) and porcine (A0541) muscle actin, platelet factor 4 (PF-4), and protamine were from Sigma; basic fibroblast growth factor (bFGF), tumor necrosis factor α (TNF-α), and transforming growth factor β (TGF-β) were from Genzyme; Na^{125}I was from DuPont/New England Nuclear; and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide was from Pierce. A polyclonal rabbit anti-actin antiserum (A2668) was purchased from Sigma. Its IgG fraction was prepared by protein A-Sepharose chromatography. Normal rabbit IgG was prepared similarly.

Isolation of AngBP. GM7373 cells (8) were cultured and used to prepare AngBP as described (6).

Binding of Angiogenin to Actin. *In solution.* Actin either in depolymerization buffer (2 mM Tris, pH 8.0/0.2 mM $CaCl_2$/0.2 mM ATP/0.2 mM dithiothreitol) or in phosphate-buffered saline (PBS), was mixed with angiogenin or ^{125}I-labeled angiogenin. The mixture was incubated at room temperature for 30 min and then 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide was added at a final concentration of 10 mM. After another 30-min incubation, the reaction was quenched by the addition of 0.1 vol of 1.0 M Tris·HCl (pH 8.0). The crosslinked complex was separated from free actin and angiogenin by SDS/PAGE and visualized by silver staining or autoradiography.

Affinity chromatography. An 8 × 20 mm column of angiogenin-Sepharose on which angiogenin (0.5–1 mg) was immobilized (6) was equilibrated with PBS, and 1 ml of actin (0.2 mg/ml) in the same buffer was applied at a flow rate of 20 ml/hr. Bound actin was subjected to the same procedure of washing and elution as described above for the purification of AngBP.

Membrane blotting. Actin was separated either by SDS/PAGE or by two-dimensional electrophoresis and electrotransferred onto a poly(vinylidene difluoride) microporous membrane. The membrane was blocked by overnight agitation at 4°C in rinse buffer (10 mM Tris·HCl, pH 7.5/150 mM NaCl/1 mM EDTA/0.02% Triton X-100) containing 5% (wt/vol) bovine serum albumin. The membrane was then incubated with ^{125}I-labeled angiogenin (10^6 cpm/ml) in rinse buffer containing 0.3% bovine serum albumin at 4°C overnight with shaking. After three washes in the rinse buffer, the membrane was air-dried and subjected to autoradiography.

Biological Assay. Angiogenesis was measured on the CAM by the method of Knighton *et al.* (9) as described (1). Potential inhibitors were mixed with angiogenin 15 min prior to application onto the discs.

RESULTS

Purification of AngBP. AngBP was purified to electrophoretic homogeneity by the procedures described above. HPLC

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Abbreviations: AngBP, angiogenin binding protein; CAM, chorioallantoic membrane; bFGF, basic fibroblast growth factor; TNF-α, tumor necrosis factor α; PF-4, platelet factor 4; TGF-β, transforming growth factor β.

(C_4 column) of the eluate from the angiogenin-Sepharose column gave four major peaks with retention times of 27, 31, 35 and 40 min (Fig. 1). The 27-, 31-, and 35-min peaks contained proteins of 15 kDa, 67 kDa, and 42 kDa, respectively, by SDS/PAGE. No protein was detectable in the 40-min peak fraction by either SDS/PAGE or amino acid analysis. Binding and crosslinking assays with ^{125}I-labeled angiogenin indicated that the 42-kDa protein was AngBP (data not shown). The nature of the 15-kDa and 67-kDa proteins is unknown as yet, but amino acid analysis and immunodiffusion experiments with an anti-albumin antibody indicated they are not angiogenin or serum albumin.

Tryptic Peptide Mapping and Amino Acid and Sequence Analyses. Affinity-purified AngBP (40 μg) was reduced with dithiothreitol, carboxymethylated, desalted, and digested overnight with 5% (wt/wt) trypsin. The tryptic peptides were separated by HPLC (C_{18} column) and a single seemingly well-resolved peak was selected for sequence analysis. Specific assignments could not be made for the first few residues but thereafter the sequence became clear as Xaa-Xaa-Xaa-Xaa-Pro-Asp-Gly-Gln-Val-Ile-Thr-Ile-Gly-Asn. A search for identities via the Protein Identification Resource at the National Biomedical Research Foundation revealed complete identity with residues 243–252 of bovine smooth muscle actin.

Tryptic peptide mapping was performed in parallel with 20 μg of C_4-purified AngBP or bovine skeletal muscle actin. Similar peptide patterns were obtained with no obvious extraneous peptide peaks (Fig. 2). Most fractions from the peptide map of AngBP were sequenced: many of them could be sequenced completely. Amino acids covering ≈40% of the entire protein were sequenced and corresponded exactly to seven regions of bovine actin, residues 21–61, 92–105, 119–128, 148–169, 178–188, 239–254, and 329–335. The presence of Thr-103, Leu-153, and Asn-162 identifies this protein as a muscle-type actin (10). No difference in sequence from known actins was apparent.

Table 1 lists a typical amino acid analysis of C_4-purified AngBP. It is close to that obtained for a sample of bovine skeletal muscle actin except for the high leucine/isoleucine ratio and the low tyrosine and methionine content. The actual composition of bovine skeletal muscle actin, based on its primary structure, is also shown. The composition of AngBP is even closer to that for bovine brain capillary actin (Table 1), which differs substantially in tyrosine and leucine content (11). Bovine smooth muscle actin composition is also listed for comparison.

Binding of Angiogenin to Actin. The binding of ^{125}I-labeled angiogenin to either bovine or porcine muscle actin was investigated by crosslinking followed by gel electrophoresis.

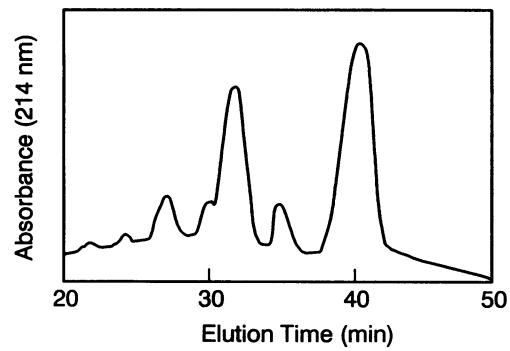

FIG. 1. Reversed-phase HPLC of AngBP. SDS eluate from the angiogenin-Sepharose column was acidified with trifluoroacetic acid to a final concentration of 0.1% and subjected to HPLC reversed-phase separation on a Supelco C_4 cartridge column. The peak at 35 min contained AngBP and was used for amino acid and sequence analyses.

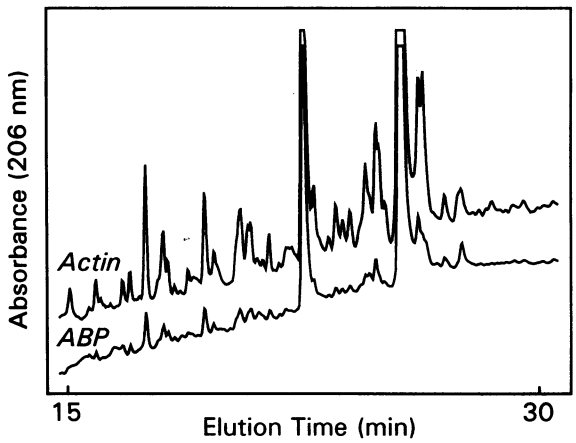

FIG. 2. Tryptic peptide map of actin and AngBP. Aliquots of bovine muscle actin (25 μg) and of AngBP (20 μg) were dissolved in 100 μl of 1% hydrogenated Triton X-100/10% (vol/vol) acetonitrile/0.3 M ammonium bicarbonate and digested with 1 μg of trypsin for 24 hr at room temperature. The peptide mixtures were dried under a stream of nitrogen, redissolved in 50 μl of 0.1% trifluoroacetic acid, and applied to a C_{18} reversed-phase HPLC column. Elution was achieved with a 1-hr linear gradient from 100% solvent A to 100% solvent B; solvent A was 0.1% trifluoroacetic acid, and solvent B was 80% acetonitrile/0.08% trifluoroacetic acid.

Autoradiography reveals a 58-kDa band in both cases (Fig. 3*a*), indicating formation of a 1:1 complex between ^{125}I-labeled angiogenin and actin. Crosslinking of nonlabeled angiogenin to actin was also examined by mixing equal amounts of the two proteins in PBS and adding the crosslinking reagent (Fig. 3*b*). After SDS/PAGE and silver staining, a 58-kDa species was detected (lane C) in addition to non-crosslinked angiogenin (16 kDa) and actin (42 kDa). The 58-kDa species was not observed in control lanes B or D where only angiogenin or actin was present.

Table 1. Amino acid compositions of bovine AngBP and bovine skeletal muscle, brain capillary, and smooth muscle actins

	Residues, no.			
		Bovine actin		
Amino acid	AngBP	Skeletal muscle	Brain capillary	Smooth muscle
Asp	34.5	33.2 (34)	35	33
Glu	43.7	37.4 (39)	47	40
Ser	27.8	22.5 (23)	28	26
Gly	38.1	30.6 (28)	36	28
His	6.6	8.0 (9)	7	9
Arg	22.2	19.3 (18)	23	18
Thr	20.1	27.1 (28)	22	24
Ala	28.0	28.8 (29)	29	29
Pro	19.3	19.7 (19)	19	19
Tyr	8.2	15.5 (16)	17	16
Val	18.8	18.3 (21)	18	20
Met	6.9	16.0 (16)	5	15
Ile	16.2	24.8 (29)	17	30
Leu	38.8	26.1 (26)	30	27
Phe	11.9	12.0 (12)	13	12
Lys	22.6	19.7 (19)	20	19
Cys	ND	ND (5)	ND	6
Trp	ND	ND (4)	ND	4

Residue numbers calculated for a molecular weight of 42,000. Numbers in parentheses are residues per molecule based on the sequence of skeletal muscle actin (10). The numbers for capillary actin are recalculated from ref. 11 and those for smooth muscle actin are from ref. 10. ND, not determined.

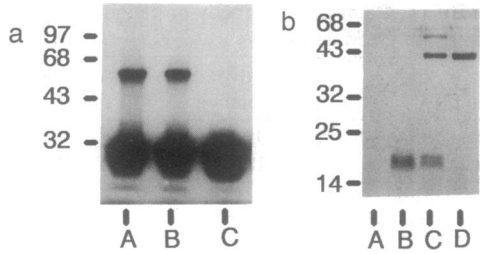

FIG. 3. SDS/PAGE analysis of binding and crosslinking between angiogenin and actin. Binding was carried out by incubating 50 ng of actin in 50 μl of PBS with 10^6 cpm of ^{125}I-labeled angiogenin (lane A) or 1 μg of actin with 1 μg of angiogenin (lane B) at room temperature for 30 min. Crosslinking was performed by incubating the mixtures of actin and angiogenin with 10 mM carbodiimide at room temperature for 30 min. The reactions were quenched with 100 mM Tris (pH 8.0; final concentration). (*a*) Autoradiography. Lanes: A, bovine muscle actin; B, porcine muscle actin; C, no actin is present. (*b*) Silver staining. Lanes: A, blank; B, angiogenin only; C, angiogenin plus bovine muscle actin; D, bovine muscle actin only.

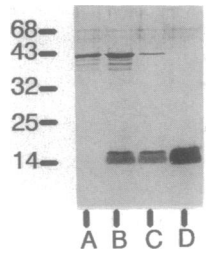

FIG. 4. Polymerization of actin by angiogenin. Angiogenin (5 μl of a 2.78 mg/ml solution) in water was added to 10 μg of bovine muscle actin in 100 μl of depolymerization buffer and mixed several times by pipetting. Polymerized actin was collected by centrifugation at 15,600 × *g* for 15 min. The supernatant and precipitate were subjected to SDS/PAGE and silver staining. Lanes: A, actin only; B, the precipitate of the actin/angiogenin mixture; C, the supernatant of the actin/angiogenin mixture; D, angiogenin only.

Increasingly higher amounts of ^{125}I-labeled angiogenin were mixed with a fixed amount of actin and crosslinked as with AngBP (6). Actin was also incubated with a fixed amount of ^{125}I-labeled angiogenin plus increasingly higher concentrations of unlabeled angiogenin and was then crosslinked. In both experiments, the ^{125}I-labeled angiogenin–actin complexes were separated from free ligands by SDS/PAGE. The gel slices that contained ^{125}I-labeled angiogenin–actin complexes were located by autoradiography, cut out, and assayed for radioactivity. A plot of bound vs. free ^{125}I-labeled angiogenin demonstrated that binding to actin reaches a maximum and is inhibited by excess unlabeled angiogenin. Scatchard analysis (not shown) based on the calculations and assumptions previously described (6) yields an apparent K_d value of 0.5 nM for the binding of actin and angiogenin, identical to that obtained for the binding of AngBP and angiogenin (6).

The binding of actin to angiogenin-Sepharose was carried out under the same conditions used for purification of AngBP by affinity chromatography. As judged by SDS/PAGE and protein staining, >90% of the applied actin binds to the column (data not shown). Actin also bound to the affinity column when equilibrated with actin depolymerization buffer. Bound actin could only be eluted by 3% SDS containing 0.5% 2-mercaptoethanol. The angiogenin–actin complex could not be dissociated by 3 M NaCl or H_2O.

Binding of ^{125}I-labeled angiogenin to actin was also analyzed by ligand blotting. ^{125}I-labeled angiogenin was found to bind to membrane-immobilized actin (data not shown).

Polymerization of Actin by Angiogenin. Many actin-binding proteins either promote or inhibit actin polymerization (12). Hence the effect of angiogenin on actin polymerization was examined. When angiogenin is mixed with globular actin at 0.2 mg/ml in depolymerization buffer, which is below the critical concentration of actin for spontaneous polymerization (0.5 mg/ml), visible turbidity develops within seconds due to the formation of aggregates. The aggregates sediment readily on microcentrifugation, stain strongly with BODIPY FL phallacidin (Molecular Probes), exhibit a needle-like structure under light microscopy (data not shown), and resolve into two bands corresponding to actin and angiogenin on SDS/PAGE (Fig. 4).

The catalytically inactive angiogenin mutants H13A and H114A (13) induced the polymerization of actin as effectively as native angiogenin. However, the putative receptor-binding-site-modified derivatives of angiogenin Ang-K and Ang-E (14) were qualitatively less capable of inducing polymerization as shown by band staining on SDS/PAGE. These observations are consistent with previous evidence that Ang-K and Ang-E fail to compete efficiently for the binding of angiogenin to AngBP (6).

PF-4 and Protamine Inhibit Angiogenin Binding to Actin. Two known angiogenesis inhibitors, PF-4 and protamine, were examined for their effect on angiogenin binding to actin (Fig. 5). The crosslinking of ^{125}I-labeled angiogenin and actin was completely inhibited by the presence of PF-4 (0.4 μg/ml) or protamine (10 μg/ml), respectively.

^{125}I-Labeled Actin Binds Other Angiogenic Factors. The observations that angiogenin binds to actin and that PF-4 and protamine inhibit the binding raise the question of whether binding to actin is characteristic of angiogenic molecules. To examine this possibility, direct binding and crosslinking experiments of ^{125}I-labeled actin to bFGF, TNF-α, and TGF-β were performed. Fig. 6 shows that ^{125}I-labeled actin is able to bind to and form complexes with bFGF and TNF-α, as established by crosslinking. ^{125}I-labeled actin does not crosslink with TGF-β under the conditions described.

Actin Inhibits the Biological Activity of Angiogenin. The ability of actin to inhibit angiogenin's biological activity was assessed by the chicken CAM assay (Table 2). In five sets of assays, 10 ng of angiogenin alone induced a positive response in 48% of the eggs (P = 0.00003), consistent with its dose–response results in previous studies (1). In the presence of 300 ng of actin, the activity of this much angiogenin was reduced to 36%, which is a statistically significant (P = 0.012) positive response. With a 100-fold molar excess of actin (3 μg), however, the effect of angiogenin in this assay was completely abolished (P = 0.196). Actin alone at either concentration did not induce significant angiogenesis.

Anti-Actin Antibody Inhibits the Biological Activity of Angiogenin. The involvement of actin in the induction of angiogenesis by angiogenin was tested by determining whether anti-actin antibodies interfere with this process. Indeed, a 10-fold molar excess of an anti-actin antibody inhibits (P = 0.29) the angiogenesis induced by 10 ng of angiogenin (Table 3). Anti-actin alone does not stimulate angiogenesis (P = 0.58) and nonimmune control IgG is not inhibitory at this level (data not shown).

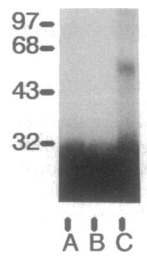

FIG. 5. Inhibition by PF-4 and protamine of the crosslinking of ^{125}I-labeled angiogenin to actin. Bovine muscle actin (50 ng in 50 μl of PBS) was incubated with 10^6 cpm of ^{125}I-labeled angiogenin in the presence of PF-4 (0.4 μg/ml; lane A) or protamine (10 μg/ml; lane B). Lane C was a control without inhibitors. Crosslinking was performed as described in Fig. 3.

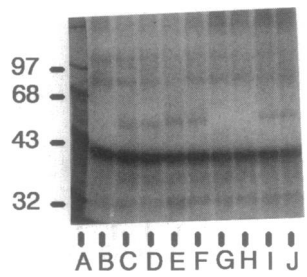

FIG. 6. Affinity crosslinking of ^{125}I-labeled actin to various angiogenic molecules. ^{125}I-labeled actin (10^6 cpm) was incubated with 0.25 μg of an angiogenic molecule in 50 μl of PBS at room temperature for 30 min and crosslinked with carbodiimide at a final concentration of 10 mM. The reactions were quenched by Tris (pH 8.0) at a final concentration of 100 mM. The mixtures were separated by SDS/PAGE and subjected to autoradiography. Lanes: A, molecular weight standards; B, ^{125}I-labeled actin only; C and D, angiogenin; E and F, bFGF; G and H, TGF-β; I and J, TNF-α. The predicted molecular masses for actin–angiogenin, actin–bFGF, actin–TGF-β, and actin–THF-α are 56, 58, 59, and 54 kDa, respectively.

DISCUSSION

Actin is a ubiquitous molecule that plays an important role in cell structure, cell motility, and generation of contractile force in both muscle and nonmuscle cells. It is found in the cytosol of all cells from amoebae to humans. It accounts for 5–10% of the total protein content of eukaryotic cells and up to 20% of the protein in muscle. Multiple forms of actin or isoactins are found in many organisms. Mammals and birds synthesize six isoforms in a tissue-specific fashion (15), suggesting that there may be a functional basis for the actin isoform multiplicity although the sequences are remarkably conserved (10, 15).

Amino acid composition, tryptic peptide mapping, and sequence analysis indicate that AngBP is a member of the actin family. Indeed, its composition is closely similar to that of the most abundant protein in bovine brain microvessels, which was also identified as a form of actin by the same techniques. This latter protein was localized to the plasma membrane of the capillary endothelial cells by immunohistochemical staining (11). The differences in amino acid composition between AngBP and any of the bovine actins (Table 1) are minor and most that have been noted may be associated with the conditions for acid hydrolysis. The close similarity of the actin and AngBP peptide maps (Fig. 2) suggests a correspondingly close similarity in sequence. Thus, it remains to be established whether or not AngBP represents another form of actin or is identical to one already characterized.

Table 2. Effect of actin on the activity of angiogenin in the CAM assay

Sample	No. positive eggs/ no. total eggs	% positive	*P*
Angiogenin (10 ng)	46/95	48	0.00003
Actin (300 ng)	16/75	21	0.598
Actin (3 μg)	13/69	19	0.824
Angiogenin (10 ng) + actin (300 ng)	27/75	36	0.012
Angiogenin (10 ng) + actin (3 μg)	19/71	27	0.196

Combined data from five sets of assays each employing between 17 and 20 eggs. The significance was calculated from χ^2 values, based on comparison with water control samples tested simultaneously, which produced 18% positive responses (14/78). $P < 0.05$ indicates a positive sample. The amount applied per egg is shown in parentheses.

Table 3. Effect of anti-actin antibody on the activity of angiogenin in the CAM assay

Sample	No. positive eggs/ no. total eggs	% positive	*P*
Angiogenin (10 ng)	35/65	54	0.00013
Anti-actin (1 μg)	18/77	23	0.58
Angiogenin (10 ng) + anti-actin (1 μg)	18/65	28	0.29

Combined data from five sets of experiments employing between 9 and 27 eggs. The significance was calculated from χ^2 values, based on comparison with water control samples tested simultaneously, which produced a 19% positive response (10/52). The amount applied per egg is shown in parentheses.

We had reported (6) that AngBP is a cell surface protein that can be detached from the cell by heparan sulfate and, probably, by angiogenin. Immunofluorescent staining of cultured endothelial cells with anti-actin antibodies reveals the presence of a smooth muscle type of α-actin on the cell surface (J. Moroianu, J.W.F., J.F.R., and B.L.V., unpublished data). Exposure of the cells to angiogenin diminishes the cell surface staining with anti-actin antibodies, supporting the conclusion that AngBP is a dissociable cell surface actin. We cannot exclude the possibility, of course, that angiogenin also reacts with actin intracellularly.

Actin has been reported to be present on the surface of normal B lymphocytes (16, 17) and to be a constituent of the intercellular matrix of smooth muscle (18), fibroblasts (19), and endothelial cells (20). Furthermore, it is distributed along the vascular internal elastic membrane in small arterioles and capillaries as shown by immunohistological staining (21). The present data provide further clues for understanding the physiological significance of cell surface actins.

Angiogenin induces the polymerization of actin. This has precluded direct measurements of binding by standard spectral or fluorescent means. Nevertheless, it may relate to the physiological mode of actin of angiogenin. We have reported (6) that AngBP (actin) appears to be released from the cell surface of cultured endothelial cells by addition of angiogenin to the medium. Indeed, it might be that induction of polymerization on binding of angiogenin to cell surface AngBP causes it to detach from the cell surface. Cell surface actin of cultured endothelial cells likely corresponds to extracellular matrix (ECM)–actin *in vivo*. Hence, binding of angiogenin to ECM–actin molecules could lead to cell detachment from the ECM followed by migration, proliferation, and differentiation into microvessels, all of which are components of the angiogenic process. Importantly, reorganization of extracellular actin has been observed during the growth and formation of the corneal endothelium (22).

Angiogenin is a member of the ribonuclease superfamily with 33% sequence identity to bovine pancreatic RNase A and an overall sequence similarity of ≈53%. All of the major components of the active site of RNase A are conserved and angiogenin exhibits ribonucleolytic activity, which although greatly diminished compared to that of RNase A, is nevertheless essential to its angiogenic activity. In addition the nonactive site region of angiogenin that encompasses residues 60–68 has been implicated as part of a cell surface receptor binding site that is also crucial for angiogenic activity (14). This region is relatively well conserved among the angiogenins from various species but differs markedly from the pancreatic RNases. It is notable that none of these RNases is angiogenic and only bovine seminal RNase, a dimeric protein with a number of unusual biological properties, has been reported to be an actin-binding protein, a property that is lost on monomerization (23).

The modified angiogenins Ang-K and Ang-E, whose putative cell-surface binding sites have been disrupted by

limited proteolysis (14), are much weaker inducers of the polymerization of actin than native angiogenin or the catalytic active site mutant angiogenins H13A and H114A. This suggests that the binding of angiogenin to actin involves its cell surface binding region and is consistent with the proposed dual site model for angiogenin (14).

A large number of proteins can bind to actin and function in the nucleating, capping, stabilizing, severing, bundling, and mechanical movement of actin filaments (12). Through the interactions with these proteins, actin filaments participate in several essential cellular functions, including cell motility, cytokinesis, maintenance of cell structure, and organelle movement (12). Angiogenin can be added to the growing list of actin binding proteins. One of the earliest changes in the cytoskeleton after certain ligands bind to their receptors is the polymerization of globular actin to form filamentous actin (24). An indirect association between receptor binding and rapid polymerization of actin has been demonstrated in several receptor systems on various cell types (25). A direct reaction between angiogenin and actin could be more effective. Castellani and O'Brien (26) reported that the biologically active form of nerve growth factor interacts with actin, which becomes organized into well-ordered paracrystalline arrays. They suggested that the *in vitro* formation of nerve growth factor–actin complexes may be related to the *in vivo* mechanism of action of nerve growth factor.

The crosslinking of angiogenin to actin is inhibited by protamine, a 4.3-kDa arginine-rich basic protein known for its capacity to inhibit *in vivo* angiogenesis induced by various angiogenic stimuli (embryologic, neoplastic, inflammatory, and immunogenic) (27). Another angiogenesis inhibitor, PF-4 (28), also inhibits the crosslinking of angiogenin to actin. The finding that *in vivo* angiogenesis inhibitors can antagonize ligand–receptor (or ligand-binding protein) interactions is important and implies that the binding of angiogenin to actin may have physiological significance.

It is not clear as yet whether actin is the functional receptor that is responsible for the cellular responses to angiogenin observed in cultured endothelial and smooth muscle cells (2–5) and for the *in vivo* neovascularization induced by angiogenin. However, the data suggest that the binding of angiogenin to actin may be an essential step. In particular the inhibitory effect of actin and anti-actin antibodies on angiogenin-induced angiogenesis in the CAM assay support this hypothesis. It cannot be ruled out that this inhibition has a more complex basis including but not solely related to the polymerizing effect of angiogenin on actin.

Two widely distributed multifunctional growth factors, bFGF and TNF-α, are angiogenic molecules with distinct cellular receptors for different cellular responses. Stimulation of angiogenesis induced by these molecules *in vivo* is thought to occur via binding to these receptors, which have been identified from cells in culture. The fact that both bFGF and TNF-α can bind to actin suggests that this may reflect a more general mechanism induced by different angiogenic molecules and perhaps provide a common pathway of angiogenesis. Indeed the ubiquitous distribution of actin may provide a convenient and effective way to regulate angiogenesis. Moreover, the inhibitory activity of actin on angiogenesis induced by angiogenin in the CAM suggests a class of angiogenesis inhibitors that may have therapeutic benefit in pathological conditions characterized by excessive blood vessel growth.

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