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Original Paper

Binding of human angiogenin inhibits actin polymerization

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ABSTRACT

Angiogenin is a potent inducer of angiogenesis, a process of blood vessel formation. It interacts with endothelial and other cells and elicits a wide range of cellular responses including migration, proliferation, and tube formation. One important target of angiogenin is endothelial cell-surface actin and their interaction might be one of essential steps in angiogenin-induced neovascularization. Based on earlier indications that angiogenin promotes actin polymerization, we studied the binding interactions between angiogenin and actin in a wide range of conditions. We showed that at subphysiological KCl concentrations, angiogenin does not promote, but instead inhibits polymerization by sequestering G-actin. At low KCl concentrations angiogenin induces formation of unstructured aggregates, which, as shown by NMR, may be caused by angiogenin's propensity to form oligomers. Binding of angiogenin to preformed F-actin does not cause depolymerization of actin filaments though it causes their stiffening. Binding of tropomyosin and angiogenin to F-actin is not competitive at concentrations sufficient for saturation of actin filaments. These observations suggest that angiogenin may cause changes in the cell cytoskeleton by inhibiting polymerization of G-actin and changing the physical properties of F-actin.

Introduction

The mechanism of action of angiogenin, a potent angiogenic factor, is not yet fully understood, but it appears to involve several different pathways from receptor binding on endothelial cells and nuclear transport to activation of proteolytic enzymes and cascades [1,2]. The small, 14 kDa protein is homologous to the much-studied bovine pancreatic RNase A [3] and its ribonucleolytic activity, albeit several orders of magnitude weaker than the ribonucleolytic activity of RNase A [4,5], is essential for its angiogenic effects [6–8] and complemented by a putative receptor binding site located within a non-catalytic region of the protein, comprising residues 58–70 and 108–111 [5,9–11]. This is evidenced by observations that if the receptor binding site is damaged or altered, these variants of angiogenin lack angiogenic functions, while the enzymatic activity remains intact. In addition, when a structurally divergent surface loop (residues 59–73) in homologous non-angiogenic RNase A was replaced with residues 58–70 of angiogenin, the recombinant protein acquired the ability to promote angiogenesis [12]. To date, a few angiogenin-binding proteins have been characterized, including actinin [13], fibulin 1 [14], follistatin [15], a 170 kDa cell surface protein with an unknown amino acid sequence expressed by endothelial cells [16] and actin [17], a protein that plays an essential role in cell movement and morphology.

Angiogenin and actin appear to form a high-affinity complex with an apparent dissociation constant of ~1–10 nM [17–19]. Attempts have been made to identify the actin-binding site on angiogenin [17,18]. For example, replacement of the active-site histidine residues His13 and His114 by alanine did not alter the capacity of angiogenin for actin binding. However, some proteolytically cleaved forms of angiogenin, e.g. at residues 60–61 or 67–68, had considerably reduced affinity for actin, which suggests that actin interacts with angiogenin via the putative receptor binding site [17,18]. The angiogenin/actin complex was found to accelerate the generation of plasmin, while angiogenin itself blocks actin's ability to inhibit the enzymatic activity of plasmin [20], which was proposed as a mechanism for angiogenin to promote invasiveness of endothelial cells [21].

Despite growing evidence for the importance of the angiogenin/actin complex for angiogenesis, little is known about the molecular details of their binding interactions. It was reported that angiogenin promotes actin polymerization [17], however, the experiments were done at low ionic strength conditions only and the structural nature of the formed polymers has not been investigated. Our studies were undertaken to examine how angiogenin binding affects the polymerization of monomeric G-actin in various conditions and whether it binds to F-actin filaments. We used a

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variety of biophysical techniques including fluorescence, differential centrifugation and electron microscopy.

Materials and methods

Protein purification

Chicken pectoral muscle skeletal actin was purified from acetone powder as described [22]. G-actin was purified on a Sephacryl S-300 column [23] and was stored in ice. Actin was labeled with pyrenyl-iodoacetamide (Invitrogen Molecular Probes, Eugene, OR), and the labeling yields were calculated according to published procedures [24,25]. The degree of the labeling was 80–99%. Recombinant short non-muscle α-tropomyosin, TM5a, and long striated muscle α-tropomyosin, stTM, purified from chicken muscle tissue were a generous gift from Dr. Sarah Hitchcock-DeGregori (RWJMS, Piscataway, NJ). The actin concentrations were calculated from the UV spectrum using an extinction coefficient of 11.0 (1% at 280 nm). Concentrations of angiogenin and tropomyosin were determined by measuring their difference spectra in 6 M guanidine–HCl between pH 12.5 and 6.0 [26] using the extinction coefficients of 2357 (M^{-1} cm^{-1}) for tyrosine and 830 (M^{-1} cm^{-1}) for tryptophan [27]. Protein purity was evaluated using SDS–PAGE [28].

Unlabeled and ^{15}N-labeled recombinant human angiogenin samples were produced in *Escherichia coli* BL21(DE3) (Novagen) grown in, respectively, LB or M9 medium containing ^{15}N-ammonium sulfate as the sole nitrogen source [29]. The expression plasmid encoding the gene of human angiogenin was a generous gift from Dr. Robert Shapiro of Harvard Medical School. Protein purification was carried out following a modified procedure for the isolation of mouse angiogenins [30]. Briefly, insoluble cell extract obtained from 2 L of cell culture was resuspended and solubilized in 7 M guanidine–HCl, 0.15 M reduced glutathione, 0.1 M Tris–HCl, 2 mM EDTA, pH 8.0, to the final volume of 40 mL, and stirred under nitrogen for 2 h. The supernatant was dialyzed against a 2 L solution of 0.5 M L-arginine–HCl, 0.6 mM oxidized glutathione, pH 8.0 for 24 h, cleared by centrifugation for 30 min, at 10,000 g, and diluted 5-fold with water. The diluted sample (250 mL) was filtered to remove newly formed insolubles, loaded on SP-Sepharose at 10 mL/min, washed with 25 mM Tris–HCl, 0.2 M NaCl, pH 8.0, and eluted with 25 mM Tris–HCl, 0.8 M NaCl, pH 8.0. The protein was purified on a Vydac C4 reverse-phase HPLC column using a 25–45%, 1%/min, acetonitrile gradient in 0.1% TFA. HPLC fractions containing purified angiogenin were dialyzed in a dialysis bag extensively against 20 mM sodium acetate, pH 5.5. The identity of the purified protein was confirmed by mass spectrometry and NMR spectroscopy. The protein samples were stored at 4 °C before use. Immediately before NMR experiments, angiogenin was exchanged into an appropriate buffer using Amicon Centriprep YM-3 filter units.

Fluorescence measurements

Actin polymerization was measured using the change in pyrene–actin fluorescence [24] using a PTI fluorimeter (Lawrenceville, NJ) (excitation, 366 nm and emission, 387 nm, with a 1 nm slit). Polymerization was monitored by the increase in fluorescence when 250 μL of the samples containing 2 μM actin (10% pyrenylactin) in the depolymerization buffer (2 mM Tris, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM dithiothreitol, 1 mM NaN_3) were mixed with an equal volume of angiogenin (0–6 μM) in 2× polymerization buffer (200 mM KCl, 2 mM EGTA, 50 mM imidazole, pH 7.0). The experiments were done both in the presence of 2 mM $MgCl_2$ and the absence of magnesium. In the salt dependence experiments, KCl concentration in the added solutions was varied from 0 to 600 mM. Each sample was prepared in sets of four, and the fluorescence was followed 60 min at 25 °C in parallel in a four-cuvette holder.

Sedimentation experiments

The experimental conditions were the same as in the fluorescence studies, and the polymerization reactions were carried out for 1–2 h before being centrifuged. The interactions of angiogenin and tropomyosin with F-actin were followed for 2 h by the addition of 0.1 M KCl to G-actin (1 μM) at 25 °C. Angiogenin and/or tropomyosin were added to F-actin to the final concentration of 1 μM and incubated for 1–2 h. Reaction mixtures (200 μL) were centrifuged at 100,000 rpm for 20 min at 4 °C. In differential sedimentation studies, samples were progressively pelleted at 15,000, 60,000 and 100,000 rpm for 20 min at 4 °C (TLA-100, Beckman). Protein ratios in the aggregates or polymers were measured by quantifying co-sedimented precipitates. The pellets were suspended in 20 μL of the SDS–PAGE sample buffer and analyzed using SDS–PAGE. The gels were stained with Coomassie R-250, and quantified using a Molecular Dynamics model 300A computing densitometer (Sunnyvale, CA). Mixtures of angiogenin and actin at known ratios were used as controls to transfer density ratios to molar ones.

Electron microscopy

The specimens were prepared by negative staining with 2% uranyl acetate on carbon-coated copper grids. A 20 μL sample drop containing 1–2 μM total protein was placed on the grid for 2 min, blotted with filter paper, and stained with 20 μL 2% uranyl acetate for 1–1.5 min. Excess stain was removed with filter paper, and the grid was air dried. Samples were examined on a Phillips CM12 electron microscope (FEI, Eindhoven, The Netherlands), equipped with a digital camera operating at 120 kV.

NMR experiments

Two-dimensional [^{15}N–^{1}H]-HSQC spectra were recorded in 20 mM sodium acetate buffer, pH 5.0 or 6.8, at 25 °C, using an Avance-800 MHz NMR spectrometer (Bruker). The solvent proton signal was suppressed by a WATERGATE pulse sequence [31]. Intermolecular interactions were followed by the HSQC spectra of ^{15}N-labeled angiogenin titrated with a concentrated solution of unlabeled angiogenin in the same buffer. Assignment of the HSQC spectra was performed as described previously [29].

Results

Influence of angiogenin on G-actin polymerization

The influence of angiogenin on actin polymerization was studied using the pyrene–actin fluorescence assay at several KCl concentrations; a concentration of 1 μM was used for both actin and angiogenin. It is known that G-actin does not polymerize at low ionic strength and increasing concentrations of KCl induce polymerization of actin [32,33]. At low ionic strength, i.e. with 0.01 M KCl, the fluorescence intensity remained constant (filled circles, Fig. 1A), while higher concentrations of KCl induce polymerization of actin reported by an increase in fluorescence intensity. Consistent with [33], doubling the salt concentration from 0.1 to 0.2 M KCl somewhat decreases the fluorescence intensity, as shown in Fig. 1A, filled inverted triangles and filled squares, respectively.

At 0.01 M KCl, the addition of angiogenin at 1:1 angiogenin/actin ratio caused a time-dependent increase of pyrene–actin fluorescence (open circles, Fig. 1A). It should be noted that even though