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Physarum Vitronectin-like Protein: An Arg–Gly–Asp-Dependent Cell-Spreading Protein with a Distinct NH_2-Terminal Sequence

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A 70-kDa protein cross-reacted with anti-bovine vitronectin was isolated from slime mold *Physarum polycephalum*. The NH_2-terminal amino acid sequence of the protein, referred to as *Physarum* vitronectin-like protein, did not share any homology with those of animal vitronectins. It had cell-spreading activity, which was specifically inhibited by an Arg–Gly–Asp (RGD)-containing peptide.

INTRODUCTION

Vitronectin is a multifunctional glycoprotein in animal blood plasma. It promotes spreading of cells through the Arg–Gly–Asp (RGD) sequence located near the NH_2-terminus of vitronectin [1]. The cell-spreading activity is competitively inhibited by RGD-containing peptides [2]. Furthermore, vitronectin modulates the activities of terminal immune complements [3] and hemostatic enzymes [4, 5]. It has a heparin-binding site toward the COOH-terminus, which is cryptic in native form but exposed after treatment with 8 *M* urea [6, 7]. Based on this property, vitronectin is easily purified from human plasma by heparin affinity chromatography in the presence of 8 *M* urea [8]. The procedure has succeeded in purifying vitronectins from plasma or sera of 14 animal species. The molecular weights of these vitronectins range from 59 to 78 kDa. All of these vitronectins have similar NH_2-terminal sequences and cell-spreading activity [9, 10].

Although knowledge of the structure and properties of vitronectins from mammalian and avian blood has accumulated, nothing is known about vitronectin from primitive organisms. Recently we have found a protein that cross-reacted with anti-bovine vitronectin antibody even in slime mold *Physarum polycephalum* [10]. To know the general structure and function of the protein, *Physarum* vitronectin-like protein, we conducted the isolation and characterization of it.

MATERIALS AND METHODS

P. polycephalum. *P. polycephalum* microplasmodia were cultured according to Daniel and Rusch [11]. Briefly, microplasmodia were grown in a sterilized solution containing 1.5% bactotrypton (Difco), 0.22% yeast extract (Difco), and 1.5% glucose supplemented with several inorganic ions and hemin in a shaking incubator at 24°C.

Isolation of Physarum vitronectin. *P. polycephalum* microplasmodia were collected from a densely grown culture of 300 ml by centrifugation at 2000 rpm for 1 min at room temperature and washed twice with 590 ml of 0.13 *M* NaCl, 5 m*M* EDTA, 10 m*M* 2-mercaptoethanol, and 20 m*M* Tris–HCl (pH 8.0). The microplasmodia were homogenized with 150 ml of the above solution containing 1 m*M* phenylmethylsulfonyl fluoride by a glass homogenizer. After being centrifuged at 10,000 rpm for 10 min, the precipitate (785 mg protein) was suspended with 140 ml of the homogenizing buffer containing 0.8% Triton X-100 for 1 h at 4°C. The Triton extract (138 ml) which had been clarified at 10,000 rpm for 10 min was dialyzed against 0.13 *M* NaCl, 5 m*M* EDTA, 0.8% Triton X-100, and 20 m*M* Tris–HCl (pH 8.0) to remove 2-mercaptoethanol and incubated with a 40-ml slurry of anti-bovine vitronectin–Sepharose 4B overnight at 4°C. The slurry was packed in a column and washed with 120 ml of 0.13 *M* NaCl, 5 m*M* EDTA, and 20 m*M* Tris–HCl (pH 8.0) with 0.8% Triton X-100 and then with 400 ml of the same solution without 0.8% Triton X-100. Bound proteins of approximately 550 μg were eluted with 0.13 *M* NaCl, 5 m*M* EDTA, 8 *M* urea, and 20 m*M* Tris–HCl (pH 8.0). The unbound fraction still contained considerable proteins to be bound to the antibody–Sepharose. The remaining proteins were recovered by repeated incubation and elution in the same way up to 10 times. The total eluates (approximately 5.5 mg protein) were pooled and dialyzed against 0.01 *M* NaCl, 5 m*M* EDTA, 8 *M* urea, and 20 m*M* Tris–HCl (pH 8.0). The dialysate was loaded on a heparin–Sepharose 4B column (bed volume of 5 ml) and eluted with the same buffer containing 0.1 *M* NaCl. The eluate (approximately 0.85 mg protein) was pooled, dialyzed, lyophilized, and separated electrophoretically on SDS–polyacrylamide gels, from which approximately 40 μg *Physarum* vitronectin-like protein was electroeluted.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting procedure. SDS–PAGE was performed according to the method of Laemmli [12] using the proteins reduced with 5% 2-mercaptoethanol. Gels were stained with silver using Ag-stain DAIICHI (Daiichi Pure Chemicals). Immunoblotting was carried out as described previously [10, 13].

NH_2-terminal amino acid sequence analysis. The eluate from heparin Sepharose chromatography was electrophoresed on SDS–polyacrylamide gels which had been cast at least 1 day in advance and subjected to preelectrophoresis with an electrode buffer containing 0.1 m*M* sodium thioglycolate for a radical scavenger [14]. Proteins separated on the gels were transferred to polyvinylidene difluoride membranes (Bio-Rad). After staining with 0.1% Coomassie blue R-250, the membrane pieces blotted with *Physarum* vitronectin-like protein were excised and subjected to NH_2-terminal microsequencing analyses with a Model 477A pulse liquid phase sequencer (Applied Biosystems) [14, 15].

Cell-spreading assay. Cell-spreading activity was determined using BHK fibroblastic cells in Grinnell's adhesion medium (150 m*M* NaCl, 1 m*M* $CaCl_2$, 3 m*M* KCl, 0.5 m*M* $MgCl_2$, 6 m*M* Na_2HPO_4, and 1

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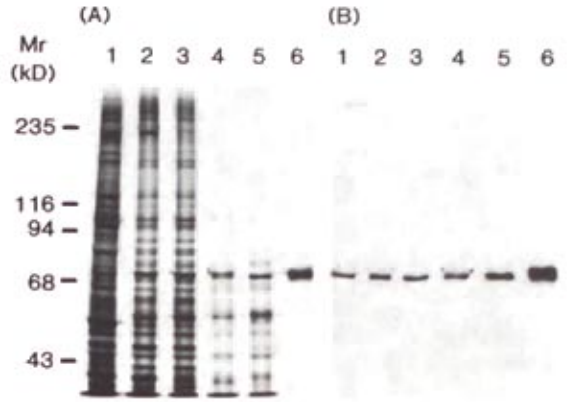

FIG. 1. Isolation of *Physarum* vitronectin-like protein. Samples of each purification step were electrophoresed on SDS-polyacrylamide gels and then stained with silver for proteins (A) or with anti-bovine vitronectin antibody (B). The homogenate of *Physarum* microplasmodia (lane 1) was centrifuged at 10,000 rpm for 10 min. The pellet (lane 2) was mixed with 0.8% Triton X-100. The Triton extract (lane 3) was subjected to anti-vitronectin-Sepharose chromatography, and the bound proteins were eluted (lane 4). The eluate was separated with heparin-Sepharose column chromatography (lane 5) and then electroeluted from preparative SDS-PAGE gels (lane 6). See Materials and Methods for details. The amounts of protein applied on the gel were 16, 5.3, 1.4, 1, 0.7, and 0.47 μg in lane 1–6 of (A) and 80, 27, 7, 1, 0.7, and 0.47 μg in lanes 1–6 of (B), respectively. Molecular size (in kDa) is indicated at the left.

m*M* KH_2PO_4, pH 7.3) [16]. Polystyrene 96-well tissue culture plates (Nunc) were coated with 50 μl of *Physarum* vitronectin-like protein, human vitronectin, or bovine serum albumin at indicated concentrations in the adhesion medium for 1 h at 37°C. Unbound protein was removed. BHK cells suspended in the adhesion medium were added to the wells (1×10^4 cells in 0.1 ml/well). After 90 min at 37°C, cells were fixed with phosphate-buffered saline containing 2% glutaraldehyde, 5% formaldehyde, and 5% sucrose and the percentage of spread cells was counted under a microscope. Inhibition assays by RGD-containing synthetic peptides were carried out on the wells coated with 1 μg/ml *Physarum* vitronectin-like protein or human vitronectin. GRGDSP or GRGESP peptides were added to the cell suspension on the wells at indicated concentrations.

RESULTS

Physarum vitronectin-like protein was detected in whole homogenate of microplasmodia as one band with a molecular weight of 70,000 (70 kDa) by immunoblotting using anti-bovine vitronectin antibody (Fig. 1, lane 1). This band was stained with neither the same antibody which had been preadsorbed with bovine vitronectin [10] nor rabbit preimmune serum (data not shown). The molecule was extracted and purified to homogeneity from *P. polycephalum* microplasmodia through four steps; extraction from the insoluble cell debris, immunoaffinity chromatography, heparin affinity chromatography, and electroelution of preparative SDS-PAGE. After the last step of purification, *Physarum* vitronectin-like protein preparations consisted of only one protein band of 70 kDa on SDS-PAGE under reducing conditions (Fig. 1A, lane 6), which also strongly reacted with anti-vitronectin antibody (Fig. 1B, lane 6). The yield of *Physarum* vitronectin-like protein was approximately 40 μg from 300 ml of culture medium of *Physarum* microplasmodia which contained 2.2 g *Physarum* proteins.

The 70-kDa band was not stained with both the same antibody preadsorbed with bovine vitronectin and rabbit preimmune serum (data not shown). However, bovine vitronectin blotted on the nitrocellulose membrane was still stained by the antibody preadsorbed with *Physarum* vitronectin-like protein. This result suggests that *Physarum* vitronectin-like protein shares some but not all the epitopes on bovine vitronectin.

Although animal plasma vitronectin is a soluble secreted protein, *Physarum* vitronectin-like protein was not detected in the conditioned medium. It was fractionated in the insoluble cell debris (Fig. 1, lane 2) but not in the soluble fraction of homogenate. Most vitronectin-like protein became extracted with 0.8% Triton X-100 (Fig. 1, lane 3), suggesting that it might be associated or enclosed with membranes. *Physarum* vitronectin-like protein was bound to heparin at 0.01 *M* NaCl, 5 m*M* EDTA, and 20 m*M* Tris-HCl (pH 8.0) in the presence of 8 *M* urea (Fig. 2B, lane 2) and at 0.13 *M* NaCl of the same buffer in the absence of 8 *M* urea (data not shown). Thus, the heparin-binding site of *Physarum* vitronectin did not seem to take urea-dependent cryptic–open conversion as those of animal vitronectins did [6, 7, 17].

The NH_2-terminal sequence of *Physarum* vitronectin-like protein is shown in Fig. 2. The sequence of 20 amino acids from *Physarum* vitronectin-like protein had no similarity to the NH_2-terminal sequences for animal vitronectins [10, 18–20] and for all proteins searched in the NBRF-PIR protein sequence database. However, the NH_2-terminal sequence was partially homologous with some intramolecular sequences of other

	1				5					10					15					20
(1)	A	S	Y	P	V I	P	Q	K	L	G	M	P D	A	L	R D	P	T S G Y	M	S	Q
(2)	D A	Q	E	S	C	K	G	R	C	T D	E Q	G	F	N	A	D T	R K	K	C	Q

FIG. 2. NH_2-terminal sequence of *Physarum* vitronectin-like molecule (1) and consensus NH_2-terminal sequence of animal vitronectins (2). Single letter amino acid abbreviations were used. In the 5th, 12th, 15th, and 17th cycles of Edman degradation of *Physarum* vitronectin-like protein, phenylthiohydantoin derivatives of two or four amino acids were detected as indicated. The consensus sequence is deduced from the identical amino acids among more than 3 of 14 animal species, except for cysteine [10]. Cysteines at the 5th, 9th, and 19th residues in the consensus sequence are from human and rabbit vitronectins [18–20].

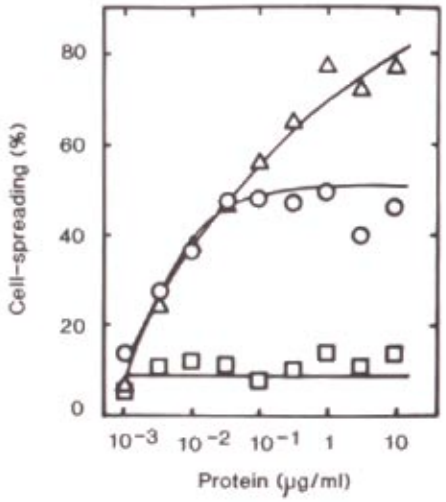

FIG. 3. Cell-spreading activity of *Physarum* vitronectin-like protein. The spreading of BHK cells was measured on the wells coated with each protein. *Physarum* vitronectin-like protein (○), human vitronectin (△), or bovine serum albumin (□) was allowed to coat the wells at indicated concentrations at 37°C for 60 min. BHK cells were added into the wells and incubated at 37°C for 90 min. The percentage of cells spread was counted under a microscope.

proteins: rat cathepsin H near the COOH-terminus (85.7–100% as for the first 7 amino acids) and *Saccharomyces cerevisiae* dihydrolipoamide acetyltransferase precursor near the NH_2-terminus (60–65% as for the 20 amino acids).

We studied whether *Physarum* vitronectin-like protein had a cell-spreading activity as human vitronectin did. Electroeluted *Physarum* vitronectin-like protein was assayed for spreading of BHK fibroblastic cells. As shown in Fig. 3, *Physarum* vitronectin-like protein was efficient in mediating spreading of BHK cells in a dose-dependent manner. *Physarum* vitronectin-like protein and human vitronectin induced a similar extent of cell-spreading up to 0.1 μg/ml. However, the activity of *Physarum* vitronectin-like protein never exceeded 55% even at the high concentrations, whereas 80% spreading was observed at 1 μg/ml of human vitronectin. The lower efficiency was not due to SDS treatment during the purification of *Physarum* vitronectin-like protein, because the cell-spreading activity of *Physarum* vitronectin-like protein in the eluates from heparin–Sepharose chromatography also showed 50% maximum cell-spreading activity and was not influenced by the treatment with SDS. The morphology of the cells spread on *Physarum* vitronectin-like protein presented slightly less expanded cytoplasm than that on human vitronectin (Fig. 4).

RGD-containing peptides are known to compete with vitronectin for binding to vitronectin receptors and they inhibit RGD-dependent cell-spreading when added exogenously [2]. Accordingly, we examined the effect of

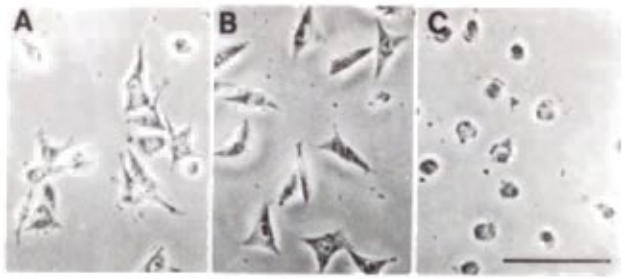

FIG. 4. Morphology of BHK cells spread on *Physarum* vitronectin-like protein (A), human vitronectin (B), and bovine serum albumin (C). Each protein was coated at a concentration of 1 μg/ml. Bar, 100 μm.

RGD peptides on *Physarum* vitronectin-like protein-mediated cell-spreading. GRGDSP peptide inhibited the cell-spreading activity of *Physarum* vitronectin-like protein as well as human vitronectin in a dose-dependent manner (Fig. 5). In contrast, a control peptide, GRGESP, did not significantly inhibit the cell-spreading activity of both *Physarum* vitronectin-like protein and human vitronectin at concentrations of up to 100 μg/ml. These results suggest the presence of a cell-spreading active RGD sequence within *Physarum* vitronectin-like protein, in common with animal vitronectins.

DISCUSSION

Although almost all studies of vitronectin have been carried out using human blood plasma vitronectin, we

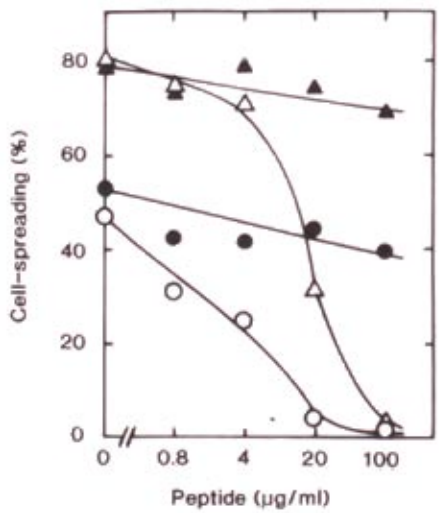

FIG. 5. Inhibition of cell-spreading activity of *Physarum* vitronectin-like protein by RGD-containing synthetic peptides. Polystyrene wells were coated with 1 μg/ml of *Physarum* vitronectin-like protein (○, ●) or human vitronectins (△, ▲) at 37°C for 60 min. The cell suspension containing GRGDSP (○, △) or GRGESP (●, ▲) peptides was added to the wells at the concentrations indicated and was incubated at 37°C for 90 min.

have searched for vitronectin from primitive organisms and have previously identified a *Physarum* protein cross-reacting with anti-bovine vitronectin antibody [10].

The immunoblotting analysis for *Physarum* proteins using the bovine vitronectin antibody revealed one band having a molecular weight of 70 kDa under reducing conditions. The 70-kDa *Physarum* vitronectin-like protein was purified to homogeneity and characterized for the first time in this paper. *Physarum* vitronectin-like protein strongly reacted with anti-bovine vitronectin antibody, but it was not detected by the antiserum preadsorbed with an excess amount of bovine vitronectin. Bovine vitronectin was still stained by the antiserum which had been preadsorbed with an excess amount of isolated *Physarum* vitronectin-like protein. These results indicate that there are some but not all common epitopes between *Physarum* vitronectin-like protein and bovine vitronectin. There are other similarities between *Physarum* vitronectin-like protein and animal vitronectin. Both had similar molecular weights and a strong affinity for heparin. Furthermore, they induced cell-spreading, which was interfered with by an RGD peptide.

However, *Physarum* vitronectin-like protein had several properties distinct from those of known animal vitronectins. The NH_2-terminal sequence of *Physarum* vitronectin-like protein was totally different from the consensus sequence of animal vitronectins. It was insoluble in the *Physarum* homogenate and recovered associated with sedimental cellular materials. The heparin-binding site of the protein was not cryptic. In addition, the cell-spreading activity was different; the plateau level of cell-spreading activity was lower.

Recently, Sanders *et al.* [21] identified a 55-kDa vitronectin-like protein in flowering plants with anti-human vitronectin. They also found that plant genomic DNA hybridizes with human vitronectin cDNA probe. Wasi *et al.* [22] also identified 14- to 17-kDa cell-spreading proteins from porcine bone, which have cross-reactivity with a monoclonal antibody to human vitronectin and a polyclonal antibody to bovine vitronectin. From these respects, *Physarum* vitronectin-like protein seems to be a family of vitronectins which are similar but not identical with mammalian or avian plasma vitronectins. More definitive evidence will be needed to identify the *Physarum* protein as vitronectin. It appears to be important whether the intramolecular sequences are homologous with these of animal plasma vitronectins and whether the DNA sequence coding the *Physarum* vitronectin-like protein has structural similarity with those of animal plasma vitronectins.

Physarum possesses neither a blood coagulation system nor an immune complemental system as do vertebrates. At present, it is not clear what the functions of *Physarum* vitronectin-like protein in *Physarum* cells are. *Physarum* vitronectin-like protein was not secreted into the culture medium and was fractionated into an insoluble form without detergent. It seems to be associated with cell membranes or pooled in cell organelles. When *Physarum* creeps on the ground or recognizes other cells, it is possible to function as an anchor or as a recognition site. Moreover, when *Physarum* is infected by bacteria, it would bind bacteria as human vitronectin does [23] and then scavenge nonimmunologically.

The RGD-sequence recognition is a universal cell-adhesion system in a variety of living organisms [24]. The function of this system has been reported not only in vertebrates but also in the other organisms: embryogenesis in *Drosophila melanogaster* [25], cell aggregation of *Dictyostelium discoideum* [26], cell adhesion of human immunodeficiency virus-1 transactivation protein, tat, [27] and adhesion of plant cells to cell walls [28]. Our data about the RGD sensitivity of *Physarum* vitronectin-like protein reinforce the generality of the RGD-recognition system.

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