Biochimica et Biophysica Acta, 1120 (1992) 1–10

BBAPRO 34135

Vitronectin diversity in evolution but uniformity in ligand binding and size of the core polypeptide

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(Received 3 September 1991)

Key words: Vitronectin; Species specificity; Cell spreading; Deglycosylation; Ferguson plot

We isolated vitronectins from the plasma or sera of 14 animal species including mouse and rat by heparin affinity chromatography. They cross-reacted with anti-vitronectin antibody and their amino terminal sequences showed strong homology. They also promoted spreading of BHK cells and were bound to heparin and collagen in the same way. Therefore, these properties appear to be essential for vitronectin function. However, the apparent molecular weights of these vitronectins varied considerably from 59 to 78 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In addition, the number of bands also varied from 1 to 3. To search for the uniformity of vitronectin polypeptide, vitronectins were deglycosylated and examined by Ferguson plot analysis. The size of the polypeptide portion of vitronectins was estimated to range from 40 to 57 kDa which was 19–26 kDa smaller than original values. Supposing a possible cleavage site at 5–13 kDa far from the carboxyl terminus, all vitronectin polypeptides were speculated to be synthesized de novo in the size range of 50–57 kDa. Proteins reacting with anti-vitronectin antibody were also detected on the immunoblot of 13 more species including *Drosophila* and *Physarum*. Almost all of these vitronectin-like proteins showed marked species-specific variations in their apparent molecular weights from 51 to 96 kDa in SDS-PAGE.

Introduction

Vitronectin (S-protein, serum spreading factor) is a glycoprotein existing in plasma and connective tissues. Vitronectin promotes attachment and spreading of a variety of animal cells onto substrata [1,2], and it seems to be the true cell-attachment factor in animal sera for tissue culture (e.g., fetal bovine serum) [3,4]. It also modulates both actions of complements [5] and thrombin in blood [6–9]. Vitronectin is bound to heparin [10], collagen [11,12], plasminogen activator inhibitor 1 [13,14,15], β-endorphin [16] and serotonin [17], as well as the integrins of animal cells [18] and platelets [19].

The complete primary structure of human vitronectin deduced from cDNA sequence indicates that the molecular mass of the vitronectin core protein is 52 kDa [20,21]. On the other hand, analyses using SDS-PAGE under reducing conditions have reported a much larger molecular weight for human whole vitronectin ranging from 75 [10,22] to 80 kDa [23]. Even considering a carbohydrate content of 5% [11] or 11% [24], which corresponds to 4 or 8–9 kDa, there remains an unsolved difference of 14–24 kDa concerning the molecular weight of human vitronectin.

In the meantime, vitronectins from human placenta [10], human platelets [25] and animal plasma of horse, porcine, bovine, rabbit and chicken [24,26,27] have been biochemically characterized partially, but almost all knowledge of vitronectin has been based on the study of human plasma vitronectin. Nothing about vitronectin molecules from small laboratory animals and invertebrates has been reported. Therefore, the present understanding of the vitronectin molecule is almost particular to human plasma vitronectin. To know the essence of the vitronectin molecule in the animal

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; Con A, concanavalin A; PNA, peanut agglutinin.

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kingdom, biochemical characterization of vitronectin from many kinds of animals is necessary. For future experimental analysis of vitronectin function in vivo, some kinds of typical small laboratory animals and invertebrates should be included, because they are obviously much more suitable than humans for manipulation.

Recently we have developed a simple method to purify vitronectin from human plasma [28] and have succeeded in its application for five animal species [24]. In line with these studies, this paper first describes the isolation or identification of vitronectins from a variety of living organisms, 27 species, including laboratory small animals and invertebrates of mouse, rat, *Drosophila* and slime mold *Physarum*. The discrepancy concerning the molecular weight of human vitronectin is also resolved by both Ferguson plot analysis on SDS-PAGE and consideration of carbohydrate contents.

Materials and Methods

Plasma, sera and living organisms

Plasma samples were obtained from human, bovine, porcine, rabbit, guinea pig, hamster, rat, mouse, chicken, goose and newt with sodium citrate as an anti-coagulant. Dog plasma and hagfish serum were kind gifts from Dr. S. Nakagawa (Nihon University School of Medicine) and Dr. S. Watabe (University of Tokyo), respectively. Sera from fetal bovine, horse, goat and sheep were tissue-culture grades purchased commercially. Blood from quail, finch *Lonchura striata*, Japanese terrapin, *Xenopus laevis*, goldfish, sea bream, loach, lamprey, blue crab, prawn, octopus, abalone, clam and ascidian were collected by cardiac puncture and allowed to stand at room temperature for 2 h, then at 4°C overnight. After centrifuging at 9000 × *g* for 5 min, the supernatants, equivalent to sera, were used. Since some plasma and sera were diluted arbitrarily, plasma was converted to sera and the protein concentrations of the sera were normalized by the absorbance of 0.550 at 280 nm using a 1 cm-path length cell at 1/100 dilution of the sera, to compare the yield of vitronectin among species.

Small living organisms were incubated in a boiling water bath for 5 min immediately after homogenizing or mixing the whole bodies with SDS-sample buffer supplemented with proteinase inhibitors of 1000 units/ml aprotinin, 20 μg/ml leupeptin, 20 mM EDTA and 1 mM phenylmethanesulfonyl fluoride. The living organisms were: sea urchin *Hemicentrotus pulcherrimus*, sperms, eggs and embryos at stages of blastula and gastrula (Dr. S. Baba, Ochanomizu University), *Drosophila melanogastar*, strain Oregon R, adults and third instar larvae (Dr. S. Chigusa-Ishiwa, Ochanomizu University), nematode *Caenohabditis elegans* adults (Dr. H. Kagawa, Okayama University), planaria adults (Mr. K. Ohto, Ochanomizu High School), *Hydra magnipapillata*, strain 105, adults (Dr. T. Fujisawa, Natl. Inst. Genetics), sponge *Ephydatia fluviatilis* adults (Dr. Y. Watanabe, Ochanomizu University), protozoa *Tetrahymena pyriformis* (Dr. T. Miki-Noumura, Ochanomizu University), slime mold *Physarum polycephalum*, sterilely cultured plasmodia (Dr. K. Murakami-Murofushi, Ochanomizu University) and amoebae of cellular slime mold *Dictyostelium discoideum*, vegetative and aggregation stages (Dr. S. Yumura, Yamaguchi University). These materials were kind gifts from the persons whose names and affiliations are indicated in the parentheses.

HepG2 cells

HepG2 human hepatoma cells were confluently grown on a 60 mm dish in a medium of 90% Dulbecco's modified Eagle's medium and 10% fetal bovine serum. After being rinsed extensively with PBS, HepG2 cells were maintained in a 35 mm dish containing 2.5 ml of serum-free medium of Dulbecco's modified Eagle's medium supplemented with 10 nM sodium selenite. The medium exposed to HepG2 cells was collected 72 h later. The remaining cells were rinsed with PBS triplicate, solubilized in a final volume of 1 ml SDS-sample buffer containing 0.1 M dithiothreitol, and boiled for 5 min.

Purification of vitronectins

Vitronectins were purified from animal plasma and sera according to our novel method [24,28] described briefly as follows. Sera passed through a heparin-Sepharose column were supplemented with a final concentration of 8 M urea. They were charged again on the heparin-Sepharose affinity column in the presence of 8 M urea. Vitronectin was specifically bound to the column and was eluted with 0.5 M NaCl in the presence of 8 M urea.

Anti-vitronectin antibodies

Polyclonal antisera against each vitronectin from human, bovine, porcine and chicken were raised in rabbits, respectively, as follows [29]. 1–2 mg vitronectins in 2 ml complete Freund's adjuvant were injected subcutaneously. Approx. 0.5 mg vitronectins in 1 ml incomplete Freund's adjuvant were injected again 4 weeks later. Blood were collected at day 10–14 after the final boost. IgG was purified from the sera through precipitation with ammonium sulfate followed by DEAE-cellulose column chromatography. M4, mouse monoclonal antibody to human vitronectin, was a kind gift from Iwaki Glass (Tokyo, Japan). M4 reacts with vitronectin relatively independently of the species [24].

Collagen-binding assay

The collagen-binding activity of vitronectins was evaluated as the amount of vitronectins bound to native type I collagen in an ELISA system as described previously [12]. Bound vitronectins were allowed to react with M4, which was detected by horseradish peroxidase-conjugated goat antibody against mouse IgG. Bovine serum albumin instead of native type I collagen and normal mouse IgG instead of M4 were used as control, respectively.

SDS-PAGE and Ferguson plot analysis

SDS-PAGE was carried out mainly according to the method of Laemmli [30] and secondarily to the method of Weber and Osborn [31]. The Laemmli system was composed of two gel layers: a stacking gel of 4% acrylamide and a separating gel of 6, 7.5, 10, 12, 13.5 or 15% (w/v) acrylamide. The relative mobility (R_f) of protein bands was measured as relative migration between the top of the separating gel and the front band of bromophenol blue. The system of Weber and Osborn was composed only of separating gel of 5, 6, 7.5, 8.5, 10 or 12.5% (w/v) acrylamide without the stacking gel.

The retardation coefficient (K_R) was calculated with varying concentrations (T) of acrylamide using Ferguson's equation [32–34].

$$\log R_f = \log R_{fo} - K_R T$$

where R_{fo} was R_f interpolated at $T = 0$. The plots of K_R versus molecular weights (M_r) were done by curve fitting.

$$M_r = a + bK_R + K_R^2$$

The standard proteins and their M_r were β-galactosidase (116 kDa), phosphorylase *b* (94 kDa), lactoferrin (80 kDa), bovine serum albumin (68 kDa), rabbit IgG heavy chain (50 kDa), ovalbumin (43 kDa), aldolase (40 kDa), thermolysin (34.4 kDa), carbonic anhydrase (30 kDa), α-chymotrypsinogen A (25.7 kDa) and soybean trypsin inhibitor (20.1 kDa).

Western blotting

Western blotting was performed essentially according to Towbin et al. [35]. The proteins separated in SDS-PAGE were electrophoretically transferred to a nitrocellulose sheet with 5 mM sodium tetraborate for 60 min at 150 mA in a semi-dry blotter Horize blot (No. AE-6670, Atto, Tokyo, Japan). The nitrocellulose sheet was blocked with 0.2% (w/v) skim milk in PBS. The antigen was detected with a mixture of rabbit polyclonal antisera (dilution 1/1000) against each vitronectin from human, bovine, porcine and chicken, and then incubated with the second antibody of goat antibody against rabbit IgG (dilution 1/1500). After further incubation with the third antibody of horseradish peroxidase-conjugated rabbit antibody against goat IgG (dilution 1/1500), horseradish peroxidase bound to the sheet was visualized with an incubation of 34 μg/ml *o*-dianisidine and 0.01% H_2O_2 in PBS.

Deglycosylation of vitronectin

All carbohydrate chains of glycoproteins were removed by a simple procedure, which was a combination of three deglycosylation procedures described by Ii et al. [36], Fan et al. [37] and Thotakura and Bahl [38]. In the procedure, neuraminidase removes sialic acids, glycopeptidase F removes N-linked saccharides, and endo-α-*N*-acetylgalactosaminidase removes O-linked asialo-saccharides. Vitronectin (120 μl, 9 μg) dialyzed against buffer A (2 mM $CaCl_2$, 0.2 mM EDTA and 50 mM sodium acetate, pH 5.6) was added to 40 μl of buffer A containing 0.4% β-mercaptoethanol and 2% SDS. After incubation in boiling water for 3 min, vitronectin solution was added to 640 μl of buffer A containing 0.1% β-mercaptoethanol and 0.8% *n*-octyl-β-D-thioglycoside. Neuraminidase *Vibrio cholerae* (8.1 μl, 8.1 mU; Calbiochem, U.S.A.), endo-α-*N*-acetylgalactosaminidase *Alcarigenes* sp. (5.1 μl, 18 mU; Seikagaku Kogyo, Tokyo, Japan), and glycopeptidase F *Flavobacterium meningosepticum* (6.8 μl, 1.4 U; Boehringer Mannheim, Germany) were mixed with the above solution and incubated at 37°C for 16 h. To collect vitronectin, the above solution was chilled and added to 91 μl of cold 100% trichloroacetic acid at 4°C overnight. After centrifugation at 9000 × *g* at 4°C for 5 min, the precipitate was solubilized with 30 μl of 2-fold concentrated SDS-sample buffer and the pH adjusted with 0.1 M NaOH to almost neutral. Its vol. was also adjusted to 60 μl with distilled water and boiled for 5 min. A 20 μl sample of each, containing 3 μg original vitronectin, was applied on SDS-PAGE and stained with Coomassie blue or two kinds of lectins.

Deglycosylation of O-linked saccharides was alternatively performed with weak alkaline solution as described by Florman and Wassarman [39]. Vitronectin was treated with various concentrations of NaOH from 5 to 100 mM at 37°C for 16h.

Lectin staining was performed on vitronectins transferred from SDS-PAGE gels onto nitrocellulose sheets as described previously [40]. The sheets were blocked with 2% bovine serum albumin and 0.05% Tween 20 in 0.15 M NaCl and 10 mM Tris-HCl (pH 7.5). Two kinds of lectins, concanavalin A (Con A) of 1 μg/ml and peanut agglutinin (PNA) of 0.5 μg/ml, conjugated with horseradish peroxidase (Seikagaku Kogyo, Tokyo, Japan) were used to react with the sheets at room temperature for 1 h and visualized with *o*-dianisidine and H_2O_2 as mentioned above. Con A detects N-lin-

ked saccharides and PNA detects O-linked asialo-saccharides of vitronectin [24].

Other methods

Cell-spreading activity was measured using BHK cells by the procedure described previously [28]. The amino acid sequence from the amino terminus was determined with a gas phase protein sequencer type 477A (Applied Biosystems, Foster City, U.S.A.). Two bands each of human and HepG2 vitronectins were quantitatively determined by two-dimensional densitometry on the Western blotted-nitrocellulose sheet as described previously [41].

Results

Vitronectins from mammalian and avian blood

We have recently developed a simple method to purify vitronectin from plasma or sera as a heparin-binding protein in the presence of 8 M urea [24,28]. The method was applied to purify vitronectin from human, rabbit, mouse, rat, hamster, guinea pig, dog, horse, porcine, bovine, goat, sheep, chicken and goose. These species were all of those from which we could obtain more than 20 ml plasma or sera. From all of them without exception, particular proteins (vitronectins as are evident from below) were purified as a heparin-binding protein, of which the SDS-PAGE patterns are shown in Fig. 1A. There appeared 1–3 bands, of which the molecular weights ranged from 59 to 78 kDa as shown in Table I. A heparin-binding protein from fetal bovine serum of tissue culture grade was similar to that from bovine adult plasma in SDS-PAGE (data not shown).

All the heparin-binding proteins, vitronectins, had collagen-binding activity. All vitronectins promoted the spreading of BHK cells at half-maximal concentration of approx. 0.1 μg/ml. The morphology of the spread cells on the vitronectin-coated dish was indistinguishable from that on an authentic human vitronectin-coated dish. Each vitronectin gave essentially a single amino terminal sequence with similar yields of amino acid in each step (Table II). The proteins had amino terminal sequences homologous to that of authentic human vitronectin [20,21,42]. The rabbit vitronectin showed the same sequence deduced from rabbit vitronectin cDNA [27]. Further, all of the bands in Fig. 1A reacted with a mixture of polyclonal anti-vitronectin antisera on the Western blotting. On the Western blotting using whole plasma or sera, the same bands as in Fig. 1A were also detected. The only two exceptions were another upper band (80 kDa) in some mouse sera and another lower band (65 kDa) in chicken plasma. Thus, purified vitronectins seemed to be almost intact in their sizes and not considerably degraded during the purification steps.

Interestingly, there were three kinds of marked species-specific variations of vitronectin. Firstly, the molecular weights of vitronectin varied considerably from 59 to 78 kDa in SDS-PAGE at 7.5% acrylamide under reducing conditions in Fig. 1A, depending on the species (Table I). Secondly, the yield of vitronectin

TABLE I

Purification yield and molecular weights of animal vitronectins

Species	Yield * (mg)	Molecular masses (kDa)						
		(a) intact **	(b) deglycosylated **	(c) carbohydrate moiety (a) – (b)	(d) Ferguson plots of (a)	(e) Ferguson plots of (b)	(f) carbohydrate moiety (d) – (e)	(g) carbohydrate moiety ***
Human	3.1–5.6	76/68	67/59	9	59/50	52/43	7	6
Rabbit	6.7–8.6	68	58	10	53	43	10	11
Mouse	3.9–5.9	(80)/71	60	11	56	46	10	
Rat	7.9	73	59	14	60	46	14	
Hamster	6.5	71	63	8	57	49	8	
Guinea pig	2.4–3.2	69	64	5	53	48	5	
Dog	0.5–0.6	71/63	64/56	7	58/50	50/42	8	
Horse	1.3–2.0	75/64	71/61	3–4	62/50	57/44	5–6	6
Porcine	2.4–4.9	59	54	5	44	40	4	5
Bovine	1.5–4.1	78/69	68/59	10	64/55	52/43	12	11
Goat	2.1	78–65	66–55	10–12	65–53	52–44	9–13	
Sheep	1.5–1.9	78–65	66–55	10–12	65–53	52–44	9–13	
Chicken	5.1–8.6	70/(65)	64	6	56	50	6	7
Goose	9.4	71	59	12	57	47	10	

* The yield presents the amount of vitronectin isolated from 100 ml equivalent of serum, of which the absorbance at 280 nm at 1/100 dilution was normalized to 0.550 using a 1 cm path length cell.

** Values are of vitronectin bands in SDS-PAGE at 7.5% acrylamide stained with Coomasie blue, except those in parentheses which are detected only on Western blotting of whole sera or plasma.

*** Values are recalculated from a previous report [24] using new molecular weights obtained by Ferguson plot analysis.

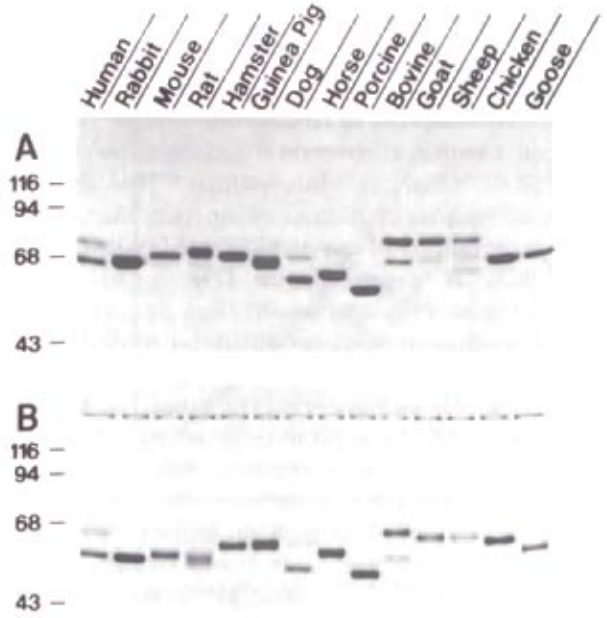

Fig. 1. SDS-PAGE of the heparin-binding proteins (vitronectins) from 14 animal plasma and sera. The heparin-binding proteins were isolated from a variety of animal plasma or sera by heparin affinity chromatography (see Materials and Methods for details). They were examined in SDS-PAGE using 7.5% (w/v) acrylamide in the separating gel after chemical reduction. Proteins in the gel were stained with Coomassie blue. Size markers (in kDa) are shown on the left. A: intact vitronectins. B: vitronectins deglycosylated under the conditions of Fig. 2, lane 4. An upper band in panel B is endo-α-*N*-acetylgalactosaminidase

per 100 ml equivalent of serum ranged widely from 0.5 to 9.4 mg, also depending on the species (Table I). A high yield of more than 6 mg vitronectin from a 100 ml equivalent of serum was obtained from rabbit, rat, hamster, chicken and goose. In contrast, dog, horse, goat and sheep resulted in a low yield of less than 2.1 mg. The yields from human, mouse, guinea pig, porcine and bovine were in between these values. Lastly, the number and proportion of bands in SDS-PAGE seemed to be species-specific. Vitronectins from human, dog, horse and bovine displayed two bands in SDS-PAGE and Western blotting. Mouse and chicken vitronectins showed one band in SDS-PAGE, but another weak band appeared on the Western blotting. Goat and sheep vitronectins were composed of three bands. All of the other animal vitronectins had one major band.

Deglycosylation of vitronectins

Vitronectin is a glycoprotein with both N- and O-linked carbohydrates, and there are striking species-specific variations of the carbohydrate composition [24]. To compare the size of vitronectin core polypeptide, vitronectins were deglycosylated with neuraminidase, endo-α-*N*-acetylgalactosaminidase and glycopeptidase F. Fig. 2 shows a typical result of sequential deglycosylation using rabbit vitronectin which is known to have both N- and O-linked saccharides [24]. Intact vitronectin (lane 1) was Con A-positive but PNA-negative, suggesting that it possessed both N-linked and sialilated O-linked saccharides. Neuraminidase-treated vitronectin (lane 2) was PNA-positive, indicating removal of sialic acids. Further treatment with endo-α-*N*-acetylgalactosaminidase (lane 3) made it PNA-negative, suggesting removal of all O-linked asialo-saccharides. Then, glycopeptidase F treatment (lane 4)

TABLE II

Amino-terminal amino acid sequences of animal vitronectins

Amino acid sequences were determined with a protein sequencer. Cysteins in the vitronectins were not identified. Small letters are slightly questionable. Enclosures indicate homology among species

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Human*	D	Q	F	S	C	K	G	R	C	T	E	G	F	N	V	D	K	K	C	Q	C	D	P	I	I
Rabbit	?	Q	F	S	?	K	L	F	?	T	E	G	I	?	S	D	?	K	?	Q	?	E	P	I	F
Mouse	D	?	?	?	?	K	G	R	?	T	Q	G	I	M	A	?	K	K	?	Q	?	D	?	L	?
Rat	Q	M	?	S	?	K	G	?	?	T	Q	G	F	M	A	?	K	K	?	Q	?	D	?	L	?
Hamster	D	Q	F	S	?	K	G	R	?	D	Q	G	F	V	A	N	R	K	?	Q	?	D	P	I	?
Guinea pig	?	?	F	S	?	K	G	R	?	T	E	G	I	N	A	D	R	K	?	Q	?	?	P	L	?
Dog	A	Q	F	S	?	K	G	R	v	T	E	G	F	N	A	D	K	K	Q	Q	Q	D	e	L	S
Horse	?	Q	E	S	?	K	D	?	?	T	E	G	F	N	A	N	W	K	?	Q	?	D	?	I	?
Porcine	?	Q	?	S	?	K	G	R	?	T	D	G	F	I	A	F	W	K	?	Q	?	E	P	I	I
Bovine	?	Q	E	S	?	K	G	R	?	T	E	G	F	?	A	T	W	R	?	Q	?	?	?	?	?
Goat	?	Q	F	S	?	K	G	R	?	T	E	G	F	?	A	T	R	K	?	?	?	?	?	?	?
Sheep	?	?	E	S	?	K	G	R	?	I	E	G	F	N	A	T	W	K	?	?	?	?	?	?	?
Chicken	a	F	D	S	K	E	G	R	?	D	E	G	F	N	A	M	K	K	?	Q	?	D	T	L	?
Goose	A	F	E	S	?	E	G	R	?	D	E	G	F	N	A	M	Q	K	?	Q	?	D	I	I	?

* Human data are taken from papers by Suzuki et al. [20] and Jenne and Stanley [21].

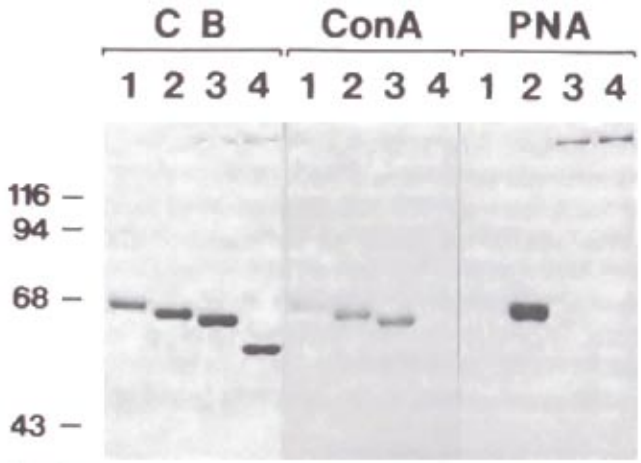

Fig. 2. Deglycosylation of rabbit vitronectin. Boiled rabbit vitronectin (3 μg) (lane 1) was deglycosylated with neuraminidase (lane 2), neuraminidase and endo-α-*N*-acetylgalactosaminidase (lane 3), and neuraminidase, endo-α-*N*-acetylgalactosaminidase and glycopeptidase F (lane 4) (see Materials and Methods for details). CB (Coomassie blue) stained for protein in the gel of SDS-PAGE at 7.5% acrylamide. Con A (concanavalin A) and PNA (peanut lectin) stained for the carbohydrate moiety of vitronectin on a nitrocellulose sheet blotted after SDS-PAGE. An upper band in lanes 3 and 4 is endo-α-*N*-acetylgalactosaminidase. Size markers (in kDa) are shown on the left.

made the vitronectin Con A-negative, indicating removal of all the N-linked saccharides. An upper band in lanes 3 and 4 was identified as endo-α-*N*-acetylgalactosaminidase. During deglycosylation steps, the molecular weights of rabbit vitronectin changed from 68, 66, 64 and finally to 58 kDa in SDS-PAGE. Thus, the carbohydrate mass of rabbit vitronectin was estimated to be 10 kDa, which was in good agreement with the 11 kDa estimated from previous chemical analysis of the carbohydrate composition [24].

Fig. 1B shows the SDS-PAGE pattern of the 14 vitronectins after the enzymatic deglycosylation. All 14 deglycosylated vitronectins were stained with neither Con A nor PNA except for weak staining of guinea pig and chicken samples with PNA, suggesting that some O-linked saccharides remained. Florman and Wassarman [39] succeeded in alkali deglycosylation of the O-linked saccharide of ZP3 with 5 mM NaOH at 37°C for 16 h without practical peptide degradation. Under similar conditions using 5–100 mM NaOH, the O-linked saccharide of chicken vitronectin was, however, not removed without peptide degradation. The intensity of PNA staining was so weak that the sizes of the remaining carbohydrate of guinea pig and chicken deglycosylated vitronectins could probably be neglected in the following analysis. All the vitronectin molecular weights, therefore, decreased with the enzymatic deglycosylation by 3–14 kDa in SDS-PAGE at 7.5% acrylamide, depending on the species (Table I). The estimated sizes of the carbohydrate moiety were in good agreement with the sizes estimated from previous chemical analysis [24].

Ferguson plot analysis of vitronectins

Purified human vitronectin migrated as two bands in SDS-PAGE (Fig. 1). Interestingly, the estimated molecular weights of human vitronectin changed with the concentrations of acrylamide in SDS-PAGE; e.g., 79/72 kDa at 6% acrylamide (Fig. 3, lane 1), but 74/61 kDa at 15% acrylamide (Fig. 3, lane 6). This type of anomalous behavior in SDS-PAGE has been already observed for other proteins including collagen [43], casein [44] and histone [33]. Since the Ferguson plot analysis for these proteins provides reliable estimates of the molecular weights, human intact vitronectin was analyzed in the same way. The relative mobility of all standard proteins interpolated at 0% acrylamide became the same value though different from that of vitronectin (data not shown). From the retardation coefficient, the molecular weights of human intact vitronectin of the so-called 75/65 kDa bands were now estimated to be 59/50 kDa with ±3 kDa, respectively (Fig. 4). They were 15–16 kDa smaller than the conventional molecular weights of 75/65 kDa. The above results were obtained in SDS-PAGE of the Laemmli system [30] and the system of Weber and Osborn [31]. Migration of both intact and deglycosylated vitronectins from 14 mammalian and avian species similarly depended on the concentrations of acrylamide in SDS-PAGE. All of their relative mobilities interpolated at 0% acrylamide were the same value though different from that of standard proteins, suggesting that both intact and deglycosylated vitronectins behaved similarly in SDS-PAGE. By Ferguson plot analysis, new molecular weights of intact vitronectins from 14 animal species were estimated to range from 44 to 65 kDa, which were 12–18 kDa smaller than those obtained in SDS-PAGE at 7.5% acrylamide (Table I). Similarly, the molecular weights of deglycosylated vitronectins were 40–57 kDa, which values were 11–17 kDa smaller (Table I). Differences in the molecular sizes between intact and deglycosylated vitronectins

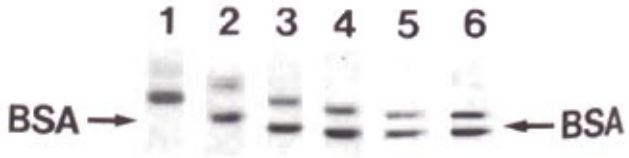

Fig. 3. Relative migration of human vitronectin in SDS-PAGE depends on the concentrations of acrylamide. Human intact vitronectin was run in SDS-PAGE using different concentrations of acrylamide in the separating gels; 6 (lane 1), 7.5 (lane 2), 10 (lane 3), 12 (lane 4), 13.5 (lane 5), and 15% (w/v) (lane 6). Arrows indicate the position of bovine serum albumin, one of the marker proteins, migrated in the same gels. Alignment of the bands is according to the migration of the band of bovine serum albumin.

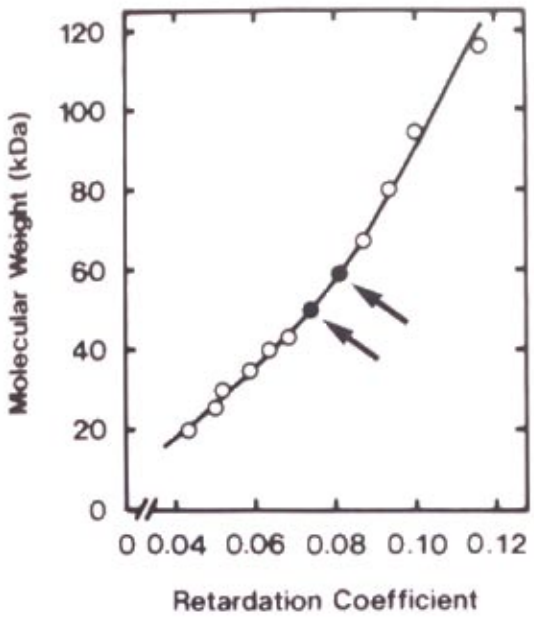

Fig. 4. Standard curve of M_r against retardation coefficient. Retardation coefficient was the slope of Ferguson plots. Open circles indicate each value of 11 standard proteins listed in Materials and Methods. Closed circles indicated by arrows show two bands of human intact vitronectin. A lower closed circle overlaps an open circle of rabbit IgG heavy chain, one of the standard proteins.

indicate the sizes of the bound carbohydrates. The differences were almost the same both before and after Ferguson plot analysis (Table I), suggesting that the estimation of carbohydrate sizes was independent of acrylamide concentrations.

Vitronectins in evolution

Anti-vitronectin antibody could detect vitronectins in 14 animal plasma or sera by Western blotting as mentioned above. Vitronectins in the other living organisms from which enough blood was not obtained were identified similarly on the blotted sheets. Fig. 5 (lane 2) shows a positive 70 kDa band in whole extracts of slime mold *Physarum* as one of the typical examples of the identification. The molecular weights of the antibody-specific bands using 7.5% acrylamide are indicated in the parentheses as follows. There were strongly stained bands in quail serum (69 kDa), finch serum (three bands between 69–74 kDa), whole *Drosophila* adults (two bands of 80 and 85 kDa) and whole nematode (two bands of 38 and 170 kDa). Weakly stained bands were detected in newt serum (96 kDa), Japanese terrapin serum (two bands of 51 and 79 kDa), *Xenopus* serum (one broad band of 58–63 kDa), goldfish serum (86 kDa), sea bream serum (three bands of 66, 68 and 86–96 kDa), hagfish serum (64 kDa), loach serum (two bands of 51–55 and 84 kDa), blue crab serum (66 kDa) and prawn serum (70 kDa). Any positive bands were neither detected for sera from octopus, clam, abalone, lamprey, ascidian, nor for the whole extracts of planaria, *Hydra*, sponge, *Tetrahymena*, yeast, cellular slime mold *Dictyostelium* (vegetative and aggregation stages), 3rd instar larvae of *Drosophila* and sea urchin (sperms, eggs and embryos at stages of blastula and gastrula).

The presence and absence of the protein reacting anti-vitronectin antibody did not correlate with phylogeny in the animal kingdom. The molecular weights of all the bands reacted with anti-vitronectin antibody except nematode ranged from 51 to 96 kDa, which were in a similar range of the mammalian and avian vitronectins shown in Fig. 1A and Table I.

Vitronectins from HepG2 cells

Barnes and Reing [45] reported that the human HepG2 hepatoma cell line is the only cell line producing vitronectin in culture among 25 cell lines. The HepG2 vitronectin is reported to migrate distinctly as one band at approx. 70 kDa, which exists between the 75 and 65 kDa bands of human plasma vitronectin in SDS-PAGE. We reexamined the migration pattern of HepG2 vitronectin by Western blotting. In contrast to the report by Barnes and Reing [45], there was nothing special in HepG2 vitronectin. Vitronectin both secreted by HepG2 cells into the culture medium and existing within HepG2 cells migrated at the same position relative to human plasma vitronectin in SDS-

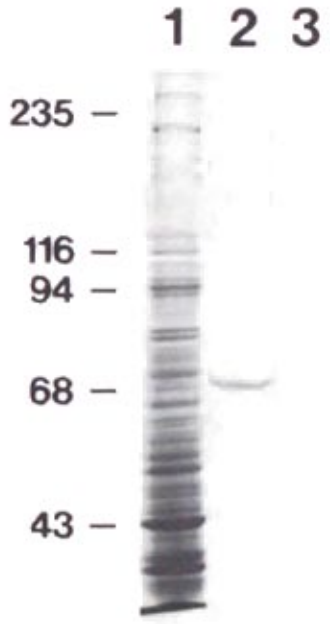

Fig. 5. Identification of the *Physarum* protein reacting with anti-vitronectin antibody. Whole proteins in slime mold *Physarum* in SDS-PAGE were stained with Coomassie blue (lane 1). The proteins electroblotted on a nitrocellulose sheet were allowed to react with anti-vitronectin antibody (lane 2) or the same antibody which had been pre-adsorbed with an excess amount of pure vitronectin (lane 3). Bound antibody to the sheet on lanes 2 and 3 was visualized with horseradish peroxidase-conjugated second antibody and *o*-dianisidine/H_2O_2. Size markers (in kDa) are shown on the left.

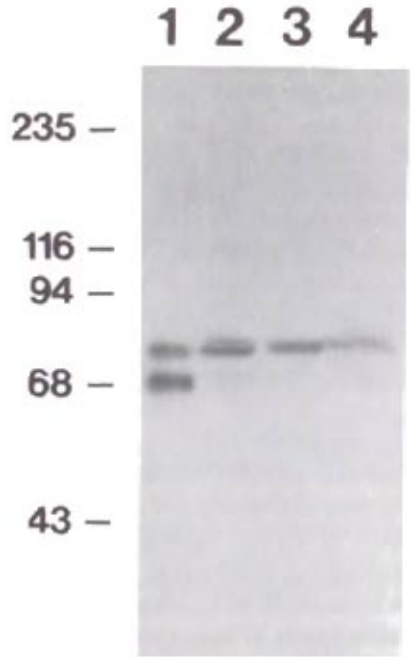

Fig. 6. Migration of HepG2 vitronectin in SDS-PAGE. Vitronectins purified from human plasma (lane 1), in original human plasma (lane 2), in conditioned medium of HepG2 cells (lane 3) and in rinsed HepG2 cells (lane 4) were detected by Western immunoblotting with anti-vitronectin antibody. Please note that there are two bands in each lane, although the lower band in lanes 2, 3, and 4 was stained very weakly. Size markers (in kDa) are shown on the left.

PAGE (Fig. 6). Besides, the so-called 65 kDa band of vitronectin was also detected in HepG2 vitronectin. From the quantitative densitometry data in Fig. 6, the ratio of the amount of two bands expressed as 65 kDa/(65 kDa + 75 kDa) was 0.13 for vitronectin secreted from HepG2 cells (lane 3), 0.21 for vitronectin within HepG2 cells (lane 4) and 0.28 for the human plasma (lane 2).

Discussion

Almost all studies on vitronectin have been focused on human plasma vitronectin since the frontier studies by Barnes, Ruoslahti and their colleagues in the early 1980's. To elucidate the general biology and biochemistry of the vitronectin molecule, however, the characterization of vitronectins from a variety of living organisms must be important. Small laboratory animals and invertebrates, for example, mouse, rat, slime mold and *Drosophila*, seem to be much more suitable for experimental manipulation of vitronectin, if they have it. We have recently developed a simple purification method for vitronectin from human plasma [28] and revealed chemical compositions and biochemical properties of vitronectin from human, horse, porcine, bovine, rabbit and chicken [24]. In this paper, animal vitronectins were isolated and characterized from 14 mammalian and avian species including small laboratory animals as well as the above six species. Furthermore, proteins reacting with anti-vitronectin were detected in 13 more living organisms of vertebrates and invertebrates including slime mold *Physarum*, nematode and *Drosophila*. All of the purified vitronectins had cell-spreading, heparin-binding and collagen-binding activities. Therefore, these functions seem to be essential for vitronectin.

The molecular weight of human whole vitronectin has been reported differently as 75 to 80 kDa by SDS-PAGE [10,22,23] and 52 kDa by cDNA sequence [20,21]. The size of the bound carbohydrates in vitronectin does not account for this difference. A similar discrepancy occurs in rabbit vitronectin [27]. With Ferguson plot analysis as well as deduction of the mass of the bound carbohydrates, the mass of deglycosylated human vitronectin of the so-called 75 kDa band was estimated to be 52 kDa in this paper, which is the same molecular weight of core polypeptide estimated from the cDNA sequence. Bound phosphate of ≈ 2.5 mol [46] and bound sulfate of ≈ 2 mol [47] per mol of vitronectin may not contribute considerably to the molecular weight estimation. Therefore, the overestimation of the molecular weights in SDS-PAGE is interpreted as a result of the abnormal behavior of vitronectin in SDS-PAGE and the bound carbohydrates.

The number and proportion of vitronectin bands in SDS-PAGE are seemingly diverse in a variety of animals. Concerning human vitronectin, the proportion of the two bands depends on the individuals [41,48] and does not seem to reflect any physiological conditions or diseases. Two bands of vitronectin are derived from a DNA polymorphism in the co-dominant alleles of the vitronectin gene, one of which synthesizes a proteinase-resistant vitronectin (corresponding to a 75 kDa band) and another synthesizes a proteinase-sensitive type (producing a 65 kDa band) [49,50]. These results may imply that the existence of the two bands is unrelated to the function of vitronectin. On the other hand, there is 11% nicked vitronectin in human plasma even when only the resistant type of the vitronectin gene is present [49]. Furthermore, two bands of vitronectin have been already observed in both secreted and cellular vitronectins in human HepG2 cells. Many species had the two-band type of vitronectin with a size difference of 5–13 kDa. They are human, mouse, dog, horse, bovine, chicken, finch, *Drosophila*, Japanese terrapin, sea bream and loach. Supposing that one of the three vitronectin bands in goat and sheep is a degradation product, then goat and sheep vitronectins are also the two-band type. Besides, one cannot exclude the possibility that all the other vitronectins are composed of two bands of which one band is too minor to be detected. Therefore, we are supposing that the occur-

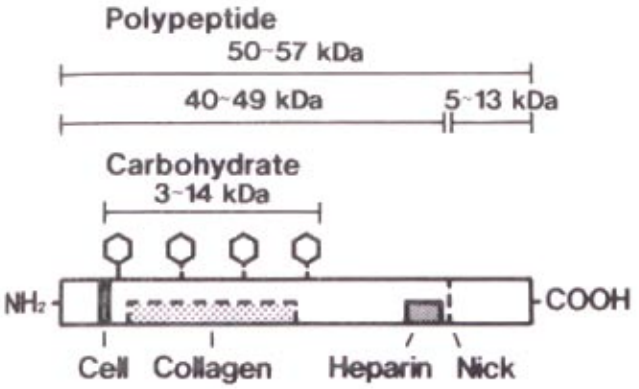

Fig. 7. A general model of vitronectin structure. A general model of vitronectin structure is proposed based on data on many animal vitronectins (this paper) and previous data on human vitronectin [12,20,21,42]. The whole core polypeptide of vitronectin uniformly spans 50–57 kDa in length with a possible cleavage site which separates it into 40–49 kDa and 5–13 kDa polypeptides. From the amino acid terminus toward the carboxyl terminus on the molecule, vitronectin commonly has a cell-spreading site of Arg-Gly-Asp, a collagen-binding site, a heparin-binding site, and a possible cleavage site (Nick). The size, number and composition of the carbohydrate chain of vitronectins are distinctly different among species.

rence of the two bands is a general property of vitronectin. This might relate to the function of vitronectin, because two bands of human vitronectin differ in heparin-binding [51], serotonin-binding [17] and phosphorylation [54]. The one-band type of vitronectin might be speculated to be either the molecule already completely cleaved or nothing cleaved.

It is tempting to speculate here on a general model of vitronectin structure as shown in Fig. 7. The whole core polypeptide of vitronectin commonly spans 50–57 kDa with a possible proteolytic site which separates it into 40–49 kDa and 5–13 kDa. There are binding sites for cells, collagen and heparin on the polypeptide. On the other hand, the size and composition of bound carbohydrate are various but species-specific.

The yields of vitronectin from different species varied considerably from 0.5 to 9.4 mg per 100 ml equivalent of serum (Table I). It is not clear what the variation reflects. The purification step of vitronectin mainly consists of two heparin affinity columns in the absence and then the presence of 8 M urea. In our previous experiments, the recovery of vitronectin from human plasma deviates from 15 to 30% [28]. The reason for the low recovery is unknown, but the recovery appears to depend on the efficiency of heparin-binding of vitronectin in the absence and then the presence of 8 M urea. We have found that very fresh human plasma contains the 2% heparin-binding form of vitronectin [51], while another group has reported the amount of 25% [52]. Conversion of fresh plasma to serum is accompanied by a 3.5-fold increase in heparin-binding vitronectin [51]. Treatment with 8 M urea remarkably increases the heparin-binding activity of human plasma vitronectin, but there still remains some heparin-unbinding vitronectin [28]. Therefore, the variation in the vitronectin yields is likely to be related to the variation in the heparin-binding properties of each animal vitronectin in the absence and then the presence of 8 M urea. One of the other possibilities is that the variation reflects the variation in vitronectin content of the samples. The variation may occur during preparation since the plasma and sera used are not necessarily collected by one person using the same protocol. One also cannot exclude the possibility that the vitronectin concentrations in circulating blood vary, but are species-specific.

Recently, Kore-Grodzicki et al. [53] found the selective phosphorylation of vitronectin in human plasma by protein kinase A. Expanding the specific phosphorylation to other animal plasma protein, they reported that phosphorylated plasma proteins of 135 kDa in rabbit, 75 kDa in guinea pig, 140, 116 and 75 kDa in rat, and 90 kDa in mouse are possibly vitronectins [54]. These molecular weights are, however, much larger than ours except for 75 kDa from both guinea pig and rat. Although we have not examined phosphorylated vitronectins, some of their proteins may not be vitronectin.

Further biochemical characterization of vitronectins from a variety of living organisms will reveal the essence of the vitronectin molecule more precisely and will provide deeper insights into the structure and function of vitronectin in the future.

Acknowledgments

We thank Ms. Kazuko Hayashi for her secretarial assistance, Dr. Tadashi Shimo-Oka for his criticism of the manuscript and Iwaki Glass (Tokyo, Japan) for anti-vitronectin monoclonal antibody. This work was supported by the Hayashi Memorial Foundation for Female Natural Scientists, by research grants from the Ministry of Education, Science and Culture of Japan and by the Special Coordination Funds of the Science and Technology Agency of the Japanese Government.

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