Yolk Vitronectin

PURIFICATION AND DIFFERENCES FROM ITS BLOOD HOMOLOGUE IN MOLECULAR SIZE, HEPARIN BINDING, COLLAGEN BINDING, AND BOUND CARBOHYDRATE*

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This is the first report on a unique vitronectin molecule, yolk vitronectin, which is similar to its blood homologue in cell spreading activity but different in molecular size, bound carbohydrate, and heparin and collagen binding activity. Yolk vitronectin was purified 2,500-fold from chick egg yolk by a combination of hydroxyapatite, DEAE-cellulose, and anti-vitronectin-Sepharose column chromatographies. In SDS-polyacrylamide gel electrophoresis under reducing conditions, yolk vitronectin was separated into 54- and 45-kDa bands, which are 16 and 25 kDa smaller, respectively, than the 70-kDa major band of chick blood vitronectin. The 54-kDa band shares the same NH2-terminal sequence as chick blood vitronectin. In contrast, the NH2-terminal sequence of the 45-kDa band is somewhat homologous with the internal sequences of mammalian vitronectins beginning at the 50th amino acid from the NH2-terminus. The bound carbohydrate of the 54- and 45-kDa species of yolk vitronectin is similar to, but distinct from, that of blood vitronectin. Unlike blood vitronectin, yolk vitronectin cannot bind to either heparin or collagen.

Vitronectin is a multifunctional glycoprotein present in human blood plasma at a concentration of approximately 0.2 mg/ml (for reviews, see Preissner (1991) and Tomasi and Mosher (1990)). It is also present in blood from a variety of animals (Kitagaki-Ogawa et al., 1990; Nakashima et al., 1992) and in human amniotic fluid, urine, platelets, monocytes, macrophages, and extracellular matrix (for reviews, see Preissner (1991) and Tomasi and Mosher (1990)). Vitronectin binds to specific receptors on fibroblasts, endothelial cells and platelets (Pytela et al., 1985, 1986), antithrombin III-thrombin complex (Jenne et al., 1985; Ill and Ruoslahti, 1985; Preissner et al., 1987), complement C5b-7 complex (Podack et al., 1978), heparin (Hayman et al., 1983; Barnes et al., 1985; Hayashi et al., 1985), collagen (Gebb et al., 1986; Izumi et al., 1988; Preissner et al., 1990; Ishikawa and Hayashi, 1992), β-endorphin (Hildebrand et al., 1988), and plasminogen activator inhibitor I (Wiman et al., 1988; Declerck et al., 1988; Seifert and Loskutoff, 1991). Vitronectin can be phosphorylated (McGuire et al., 1988; Korc-Grodzicki et al., 1988), sulfated (Jenne et al., 1989), and cross-linked by transglutaminase (Sane et al., 1988). Its physiological functions are based on the above interactions and/or modifications. Vitronectin allows cells to adhere to a substrate, promotes haptotaxis of cells (Basara et al., 1985; Naito et al., 1991), modulates thrombin and plasmin activity in fibrinolysis, and prevents thrombin lytic action of the membrane attack complex. But, the role, if any, of vitronectin in animal development and even the existence of vitronectin in embryos had not been established.

Fibronectin, a plasma glycoprotein similar to but distinct from vitronectin, also promotes cell spreading and cell migration in vitro (Yamada et al., 1976; Ali and Hynes, 1978; Rovasio et al., 1983). Extending this observation, Thiery and his colleagues have presented evidence that one of the major functions of fibronectin in vivo is related to migration of neural crest cells and gastrulation in the early development of embryos (for reviews, see Thiery et al. (1985, 1989)). Many other cell adhesion proteins, such as cadherin, laminin, and tenascin, play a role in morphogenesis in the early stages of animal development (for reviews, see Takeichi (1988), Ekblom et al. (1986), and Erickson and Bourdon (1989)). Particular combinations of these adhesion proteins and their cell surface receptors may be the key to the mechanisms by which cells are arranged to build the fine architecture of tissues during development.

We have, therefore, started to study the role of vitronectin in early development using chick embryos and anti-chicken blood plasma vitronectin. In an early stage of the experiments, surprisingly, we found that one of the egg yolk proteins reacted with anti-vitronectin. Yolk proteins are expected to influence the development of the embryo. So, we set out to characterize the yolk protein with the aim of obtaining new insight into vitronectin function in development. The yolk protein reacting with anti-vitronectin is called yolk vitronectin. In this paper, we describe its purification and a comparison of its heparin binding, collagen binding, bound carbohydrate, and molecular size with those of blood vitronectin of the same species.

EXPERIMENTAL PROCEDURES

Materials—Chick eggs were used on the day of laying or purchased commercially. Chicken blood serum and plasma were obtained from the same hen with or without the addition of a 1/7 volume of 3.18% sodium citrate as an anticoagulant and centrifuged immediately at 3,000 rpm for 10 min. For preparation of chicken blood vitronectin, pooled chicken blood plasma was obtained from Ichirei Inc. (Saitama, Japan) and stored at −20 °C until use. Chicken blood vitronectin was purified from pooled chicken blood plasma as described previously (Yatohgo et al., 1988; Kitagaki-Ogawa et al., 1990). Antibody to chicken blood vitronectin was raised in a rabbit, purified by ammonium sulfate precipitation followed by DEAE-cellulose column chro-
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Fractionation of Yolk Protein and Purification of Yolk Vitronectin—Chick eggs were divided into egg yolk and egg white. Egg yolk (18–22 g each) was suspended in an equal volume of cold 0.16 M NaCl, 2 mM phenylmethylsulfonyl fluoride, and 10 mM sodium phosphate (pH 7.4) and centrifuged at 12,000 rpm at 4 °C for 20 min (Belitcz and Grosch, 1987). The precipitate (yolk granules) was washed twice with the above solution. The supernatant (yolk plasma) was dialyzed extensively at 4 °C against 1 mM sodium phosphate (pH 7.4) containing 5 mM β-mercaptoethanol and centrifuged at 12,000 rpm at 4 °C for 20 min. Yolk plasma was separated into an upper solid layer (low density lipoprotein (LDL) fraction) and a lower soluble layer (livetin fraction). The LDL fraction was solubilized with 15 ml of 0.16 M NaCl and 10 mM sodium phosphate (pH 7.4).

Yolk vitronectin was purified from the LDL fraction by means of three chromatographic procedures on columns of hydroxylapatite, DEAE-cellulose, and anti-vitronectin-Sepharose at 4 °C as follows. The LDL fraction was applied to a hydroxylapatite column (10 ml bed volume) that had been pre-equilibrated with 0.5 M NaCl and 10 mM sodium phosphate (pH 7.4). The column was washed with 100 ml of 0.5 M NaCl and 10 mM sodium phosphate (pH 7.4) followed by 50 ml of 10 mM sodium phosphate (pH 7.4). Yolk vitronectin was eluted with 200 ml sodium phosphate (pH 7.4) from the hydroxylapatite column. The eluate was diluted with an equal volume of distilled water to decrease the ionic strength in the eluate and then applied directly to a DEAE-cellulose column (2 ml bed volume). The column was washed with 20 ml of 0.15 M NaCl, 5 mM β-mercaptoethanol, and 10 mM sodium phosphate (pH 7.4). Yolk vitronectin was eluted with 0.25 M NaCl, 5 mM β-mercaptoethanol, and 10 mM sodium phosphate (pH 7.4). The eluate from the DEAE-cellulose column was mixed and incubated with a 2 ml slurry of anti-vitronectin-Sepharose 4B at room temperature for 1 h. The slurry was packed in a column and washed with 20 ml of 0.25 M NaCl, 5 mM β-mercaptoethanol, and 10 mM sodium phosphate (pH 7.4) followed by 10 ml of 0.5 M NaCl, 5 mM β-mercaptoethanol, and 10 mM sodium phosphate (pH 7.4) and then 10 ml of 0.1 M sodium acetate (pH 4.4) containing 0.25 mM NaCl and 5 mM β-mercaptoethanol. Yolk vitronectin was eluted with 0.25 M glycine HCl (pH 2.5) containing 0.25 mM NaCl and 5 mM β-mercaptoethanol. The pH of the eluate was immediately adjusted to neutrality by adding an appropriate amount of 0.5 M sodium phosphate (pH 7.7). The antibody affinity chromatography described above was repeated several times.

Quantitation of Vitronectin and Fibrinogen—Vitronectin and fibrinogen in crude preparations were assayed by using a sandwich enzyme-linked immunosorbent assay (ELISA) with specific antibodies. For the determination of vitronectin, the wells of 96-well microtiter plates were coated with rabbit anti-vitronectin in PBS, the bound enzyme was measured using o-phenylenediamine; ConA, concanavalin A.

1 The abbreviations used are: LDL, low density lipoprotein; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; BHK, baby hamster kidney; FNA, peripheral nerve agglutinin; Lens culinaris agglutinin; PHA-L, Phaseolus vulgaris leukoagglutinin; UEA-I, Ulex europaeus agglutinin I. Allo a, Allomyrina dichotoma agglutinin; ConA, concanavalin A.

tractions of pure blood vitronectin and fibrinogen were estimated by means of absorbance measurements at 280 nm with 1-cm path length cells, using absorption coefficients of 1.38 for vitronectin (Dahlbäck and Podack, 1985) and 1.28 for fibrinogen (Mossessian and Umfleet, 1970) at 1 mg/ml. Determination of Amino-terminal Sequence—A mixture of polypeptides was separated by SDS-polyacrylamide gel electrophoresis (PAGE) using polyacrylamide gels that had been polymerized the day before and pre-electrophoresed with an electrode buffer containing 0.1 mM sodium thioglycolate to scavenge radicals (Moos et al., 1988). Proteins separated on the gels were transferred onto a polyvinylidene difluoride membrane (Immobilon-P). The amino-terminal sequence of each polypeptide on the membrane pieces was determined with a protein sequenator model 477A (Applied Biosystems) (Matsudaira, 1987). Cysteines were not identified.

SDS-PAGE, Western Blotting, Cell Blotting, and Lectin Staining—Proteins were separated by SDS-PAGE according to Laemmli (1970) and stained with Coomassie Blue. For Western blotting, cell blotting, and lectin staining, proteins separated by SDS-PAGE were transferred to nitrocellulose sheets (Schleicher and Schuell) essentially according to Towbin et al. (1979). Protein bands on the nitrocellulose sheets were observed by staining with 0.1% Amido Black 10B 45%, methanol, and 10% acetic acid for 30 s followed by washing with 90% methanol and 2% acetic acid for 30 s.

For Western blotting, the sheet was incubated with 0.2% skim milk in PBS for 30 min and then allowed to react with anti-chicken blood vitronectin antisera at 1/1,500 dilution for 1 h. Bound antibody was visualized by sequential incubation of the horseradish peroxidase-goat antibody against rabbit IgG at a 1/2,000 dilution for 1 h followed by 25 μg/ml o-dianisidine and 0.01% H2O2 for 20 min. Densitometry of the stained bands was performed as described by Kubota et al. (1988).

Cell blotting was performed according to the original report by Hayman et al. (1982) except that 0.1–0.05 μg of vitronectin/lane was used instead of 30–60 μg. BHK cells were attached to the nitrocellulose sheet at a concentration of 5 × 105 cells/ml in Grimnell’s adhesion medium at 37 °C for 90 min.

For lectin staining, proteins transferred onto separate nitrocellulose sheets were stained with 0.06–10 μg/ml each of horseradish peroxidase lectins for 1 h as described by Kitagaki-Ogawa et al. (1986). The amounts of horseradish peroxidase lectins used were sufficient to stain 6 μg of porcine vitronectin strongly as a positive control. This staining was considered to be sensitive enough to detect 1 mol of carbohydrate chain/mol of protein. The lectins used were concanavalin A (ConA), wheat germ agglutinin (WGA), Lens culinaris agglutinin (LCA), Phaseolus vulgaris leucoagglutinin L (PHA-L), peanut agglutinin (PNA), and Ulex europaeus agglutinin I (UEA-I), which were purchased as conjugates with horseradish peroxidase from Seikagaku Kogyo Inc. (Tokyo, Japan). Horseradish peroxidase-Allomyrina dichotoma agglutinin (Allo a) was from EY Laboratories (San Mateo, CA). Desialylation of vitronectin with Vibrio cholerae neuraminidase (Calbiochem), deglycosylation of O-linked asialosaccharides with endo-α-N-acetylgalactosaminidase (Seikagaku Kogyo, Tokyo, Japan), and deglycosylation of N-linked saccharides with glycopeptidase P (Boshirger & Mannheim) were carried out in 2 M CaCl2, 0.2 M EDTA, 0.1% β-mercaptoethanol, 0.8% n-octyl-β-D-thioglycoside, and 50 mM sodium acetate (pH 5.6) at 37 °C for 16 h as described by Nakashima et al. (1992).

Ligand Binding Assay—Collagen binding activity was determined by ELISA. Polystyrene microtiter plates (Sumitomo Bakelite, MS-3496F) were coated with 50 μl of native type I collagen from porcine skin (Cellmatrix-I-P, Nitta Gelatin Co., Osaka, Japan) or gelatin at 10 μg/ml in 0.1 M sodium carbonate (pH 9.6) at 37 °C for 1 h. Gelatin was prepared by boiling 0.3 mg of type I collagen/ml in phosphate-buffered saline for 5 min. After being blocked with 0.25% skim milk and 10 mM sodium phosphate (pH 7.4), the wells were washed four times with a washing solution of 0.05% Tween 20 and 10 mM sodium phosphate (pH 7.4). Various concentrations of vitronectin in 50 μl of washing solution were incubated in the wells at 37 °C for 1 h. The wells were washed with the washing solution and then immersed in immobilized protein in the wells was allowed to react with horseradish peroxidase antibody against chicken blood vitronectin diluted to 1/500 in 0.2% skim milk and 10 mM sodium phosphate (pH 7.4) at 37 °C for 1 h. The wells were washed and incubated with 100 μl of 0.1% o-phenylenediamine in 0.1% H2O2 in 0.2 M NaH2PO4 at room temperature for 10 min. Color development was stopped by adding 50 μl of 3 N H2SO4, and the absorbance at 492 nm was measured with a microtitrator plate reader, Corona MTP-32.
Identification of Vitronectin in Yolk Fractionation—A rabbit antibody to chicken blood vitronectin specifically reacted with vitronectin from chicken blood plasma in an Ouchterlony double diffusion test, ELISA, and Western immunoblotting (data not shown). Anti-chicken blood fibronectin also reacted with only fibronectin in the same tests. No cross-reaction was observed. A sandwich ELISA using these specific antibodies revealed that yolk plasma contained a fairly high concentration (0.16 mg/ml) of yolk vitronectin and a low concentration (0.03 mg/ml) of fibronectin (Table 1). The vitronectin concentration in yolk was almost the same as that in the blood plasma of the same hen. Egg white and egg yolk granules contained essentially no vitronectin or fibronectin. All the vitronectin in yolk plasma was fractionated into an upper solid layer (low density lipoprotein fraction) after extensive dialysis against 1 mM sodium phosphate (pH 7.4) followed by centrifugation at 12,000 rpm at 4 °C for 20 min. The lower soluble layer after the centrifugation, called the livetin fraction, did not contain vitronectin.

Western immunoblotting showed that vitronectin in yolk plasma migrated under reducing conditions as two bands of 54 and 45 kDa (Fig. 1, lane 6), which are 11–25 kDa smaller than those of blood vitronectin (70 and 65 kDa) (Fig. 1, lanes 1 and 4). Blood plasma also contained small amounts of vitronectin migrating at 56 and 45 kDa (Fig. 1, lane 5), which were similar in size to yolk plasma vitronectin, but they were not detected in a pure preparation of blood vitronectin (Fig. 1, lanes 1 and 4). Under nonreducing conditions, pure blood vitronectin migrated as a single band at 72 kDa (Fig. 1, lanes 7 and 10), the same position to which the major vitronectin band in blood plasma migrated (Fig. 1, lane 11), suggesting that a small polypeptide of 5 kDa may be disulfide-bonded to the 65-kDa species of blood vitronectin. On the other hand, yolk plasma vitronectin migrated as several bands at positions 54, 68, and 116 kDa (Fig. 1, lane 12), none of which co-migrated with blood plasma vitronectin of 72 kDa. These results suggest that the size of whole yolk vitronectin is different from that of blood vitronectin even if the possible existence of small disulfide-bonded polypeptides is taken into account. Yolk vitronectin may contain intramolecular disulfide bond(s) since its migration became slower under nonreducing conditions. Western immunoblotting also revealed a vitronectin aggregate at 180 kDa in blood plasma (Fig. 1, lane 11) and at 116 kDa in yolk plasma (Fig. 1, lane 12), suggesting the presence of intermolecular disulfide bond(s). The pattern of yolk vitronectin bands on Western immunoblotting was the same for chicken eggs stored at 4 °C for several days and eggs within 3 h after being laid, even when the yolk plasma from the latter eggs was prepared at 4 °C in the presence of a mixture of protease inhibitors (1,000 units/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride, 30 μg/ml soybean trypsin inhibitor, and 20 mM EDTA). Thus, the small molecular size seems to be an intrinsic property of yolk vitronectin and not an artifact resulting from degradation by yolk proteases during preparation.

Purification of Yolk Vitronectin—Yolk vitronectin was present in the solid LDL fraction of yolk plasma after centrifugation. The LDL fraction was solubilized in 0.16 M NaCl and 10 mM sodium phosphate (pH 7.4), and yolk vitronectin was purified from the soluble LDL fraction (Fig. 2, lane 3) by a sequence of three types of column chromatography: hydroxyapatite, DEAE-cellulose, and anti-vitronectin-Sepharose (Fig. 2; Table II). Yolk vitronectin was 77-fold enriched in the fraction bound to the hydroxyapatite column (Table II; Fig. 2, lane 4), and this fraction was further applied to the DEAE-cellulose column. The fraction bound (Fig. 2, lane 5) to the DEAE-cellulose column contained most of the vitronectin, giving 410-fold purification (Table II). The vitronectin preparation after the final step of antibody column chromatography was purified 2,500-fold from yolk plasma (Table II). In SDS-PAGE, pure yolk vitronectin was detected by staining with Coomassie Blue as two major bands at 54 and 45 kDa with slight contamination by small polypeptides at around 10 kDa (Fig. 2, lane 6). Only the 54- and 45-kDa bands reacted with anti-blood vitronectin on Western blotting at all stages from the starting egg yolk to the purified yolk vitronectin. The contaminating small polypeptides did not react (Fig. 2,

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**Table I**

| Egg fraction | Total protein | Vitronectin* | Fibronectin | Volume/egg |
|--------------|--------------|-------------|------------|-----------|
|              | mg/ml        | mg/ml       | mg/ml      | ml        |
| Blood plasma | 93           | 0.14        | 0.14       |           |
| Egg yolk     | (133)b       | (0.17)b     | (0.03)b    | 20.2      |
| Yolk plasma  | 101          | 0.16        | 0.03       | 15.4      |
| Livetin      | 44           | 0           | 0          | 9.2       |
| LDL          | 55           | 0.15        | 0.03       | 6.2       |
| Yolk granules| 32           | 0.01        | 0          | 3.7       |
| Egg white    | 160          | 0           | 0          | 33.0      |

*p Vitronectin concentrations are relative values estimated by ELISA using chicken blood vitronectin as a standard protein.

b Values in parentheses are the summation of values for yolk plasma and egg yolk granules.
purified sequentially from chick egg yolk (see Table II and "Experimental Procedures" for details) and subjected to protein composition analysis (lanes 1–6) by SDS-PAGE under reducing conditions and to Western blotting (lanes 7–12). Egg yolk (lanes 1 and 7) was centrifuged at 12,000 rpm for 20 min. The supernatant (yolk plasma, lanes 2 and 8) was centrifuged again after extensive dialysis against a low ionic strength solution. The upper solid layer (LDL fraction, lanes 3 and 9) was solubilized with 0.16 M NaCl and 10 mM sodium phosphate (pH 7.4). Yolk vitronectin in the LDL fraction was purified on hydroxylapatite (in kDa) is indicated at the left. The 45/54 kDa ratio ranged from 2.4 to 1, depending on the preparations, from crude egg yolk to pure vitronectin. It tended to be high in older or purer preparations, suggesting some conversion to the 45-kDa polypeptide.

**Cell-spreadening Activity of Yolk Vitronectin**—BHK cells spread on yolk vitronectin-coated microtiter plates (Fig. 3B). The shape of the spread cells was similar to that on blood vitronectin-coated plates (Fig. 3A). The dose-response curve of the cell-spreadening activity was identical for blood and yolk vitronectins, giving a half-maximal concentration of 0.03–0.1 µg/ml (Fig. 4A). A synthetic peptide GRGDSP, but not GRGESP, completely inhibited spreading of BHK cells on a yolk vitronectin-coated plate as well as on a blood vitronectin-coated plate (Fig. 4B). These results indicate that the cell-spreadening properties of yolk vitronectin are essentially the same as those of blood vitronectin.

Yolk vitronectin is a mixture of 54- and 45-kDa proteins. We examined the cell attachment activity of each yolk vitronectin band using a so-called "cell-blotting" analysis developed by Hayman et al. (1982). On nitrocellulose sheets, BHK cells were attached to the 54-kDa yolk vitronectin (Fig. 5, lane 3) as well as 70- and 65-kDa bands of chicken blood vitronectin (Fig. 5, lane 4). BHK cells, however, were not attached to 45-kDa yolk vitronectin (Fig. 5, lane 3). Protein staining of the nitrocellulose sheet (Fig. 5, lanes 1 and 2) indicates that the failure of cell attachment was not caused by a lower efficiency of transfer of the 45-kDa band to the nitrocellulose sheet. The contaminating small polypeptides at around 10 kDa also lacked cell attachment activity (Fig. 5, lane 3).

**Amino-terminal Sequence of Yolk Vitronectin**—Yolk vitronectin was separated into 54- and 45-kDa bands by SDS-PAGE under reducing conditions. These bands were transferred onto a polyvinylidene difluoride membrane, and their amino-terminal sequences were determined. We compared the results with those reported previously (Table III). Only the NH₂-terminal sequence of chicken blood vitronectin is available (Nakashima et al., 1992), whereas the whole sequences

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**TABLE II**

| Purification stage | Total volume | Total protein | Total vitronectin* | Purification fold |
|--------------------|--------------|---------------|-------------------|-----------------|
| Yolk plasma        | 140 ± 7370   | 3.53          | 1                 | 1               |
| LDL                | 26 ± 2960    | 3.12          | 2.3               | 2.3             |
| Hydroxylapatite    | 35 ± 34**    | 1.19          | 77                | 77              |
| DEAE-cellulose     | 4.0 ± 3.4b   | 0.63          | 410               | 410             |
| Anti-vitronectin-Sepharose | 4.2 ± 0.19b | 0.22          | 2500              | 2500            |

*Vitronectin concentrations are relative values estimated by ELISA using chicken blood vitronectin as a standard protein.

**FIG. 2.** SDS-PAGE and Western blotting at each step in the purification process of yolk vitronectin. Yolk vitronectin was purified sequentially from chick egg yolk (see Table II and "Experimental Procedures" for details) and subjected to protein composition analysis (lanes 1–6) by SDS-PAGE under reducing conditions and to Western blotting (lanes 7–12). Egg yolk (lanes 1 and 7) was centrifuged at 12,000 rpm for 20 min. The supernatant (yolk plasma, lanes 2 and 8) was centrifuged again after extensive dialysis against a low ionic strength solution. The upper solid layer (LDL fraction, lanes 3 and 9) was solubilized with 0.16 M NaCl and 10 mM sodium phosphate (pH 7.4). Yolk vitronectin in the LDL fraction was purified on hydroxylapatite (in kDa) is indicated at the left.

**FIG. 3.** Spreading of BHK cells on vitronectin-coated wells. BHK cells were incubated at 37 °C for 90 min on wells precoated with 3.4 µg/ml chick blood vitronectin (A), chick egg yolk vitronectin (B), or bovine serum albumin (C).

**FIG. 4.** Dose-response curve of vitronectin for cell spreading (A) and its inhibition by synthetic peptides (B). A, BHK cells were allowed to spread at 37 °C for 90 min on wells precoated with blood vitronectin (O), yolk vitronectin (O), or bovine serum albumin (Δ) at the indicated concentrations. B, BHK cell suspension containing a synthetic peptide, GRGDSP (■, ●) or GRGESP (□, ○), at the indicated concentrations was incubated on wells precoated with 1 µg/ml blood vitronectin (□, ■) or yolk vitronectin (○, ●).

**FIG. 5.** Cell blotting of vitronectin. Two pairs of pure chick yolk vitronectin (lanes 1 and 3) and blood vitronectin (lanes 2 and 4) were subjected to SDS-PAGE and transferred from the gel onto nitrocellulose sheets. One sheet was stained with Amido Black 10B for protein (lanes 1 and 2). Another sheet was examined by cell blotting using BHK cells at 37 °C for 90 min (lanes 3 and 4). Molecular mass (in kDa) is indicated at the left.
TABLE III
NH2-terminal amino acid sequences of chick yolk vitronectin polypeptides

| Amino acid sequences | 1  | 5  | 10 | 15 | 20 |
|----------------------|----|----|----|----|----|
| Yolk 54 kd band      | A   | E   | D   | S  | ?   |
| Chick blood vitronectin\(^a\) | E | G | F | N | A | M | K | K |
| Yolk 45 kd band      | E   | G   | E   | R | ?   |
| Human vitronectin\(^b\) | L | D | Y | D | S | I | D | T | G | ? | V | G | R | P | E | ? | N |
| Rabbit vitronectin\(^c\) | T | M | P | E | D | E | Y | T | V | Y | D | D | G | E | E | K | N | N | A | V | H | Q |
| Mouse vitronectin\(^d\) | T | M | P | E | D | E | Y | G | P | Y | D | Y | I | E | Q | T | K | D | N | A | S | V | H | A |

\(^a\) Nakashima et al., 1992. Small letter indicates residue identified with some ambiguity.
\(^b\) Suzuki et al., 1985; and Jenne and Stanley, 1985.
\(^c\) Sato et al., 1990.
\(^d\) Seiffert et al., 1991.

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Fig. 6. Collagen binding of vitronectin. Blood vitronectin (\(\square\)) or yolk vitronectin (\(\bigcirc\)) was incubated at the indicated concentrations on wells precoated with 10 \(\mu\)g/ml native type I collagen (\(\square\)), or heat-denatured gelatin (\(\bigcirc\)) at 37 °C for 1 h. Blood vitronectin on the collagen- or gelatin-coated wells was measured in terms of absorbance at 492 nm by means of ELISA assay with horseradish peroxidase-conjugated anti-vitronectin.

Of human, rabbit, and mouse vitronectins are known (Suzuki et al., 1985; Jenne and Stanley, 1985; Sato et al., 1990; Seiffert et al., 1991). The NH2-terminal sequence of the 54-kDa band is the same as that from chicken blood vitronectin. In contrast, the NH2-terminal sequence of the 45-kDa band is completely different, though it does have some homology with the internal sequences of mammalian vitronectins beginning at the 50th amino acid from the NH2 terminus. These results suggest that the 45-kDa band may be derived from the 54-kDa band by cleavage of the NH2-terminal 49-amino acid peptide.

Heparin and Collagen Binding Activity of Yolk Vitronectin—Fig. 6 shows that blood vitronectin binds to native type I collagen and heat-denatured gelatin. In contrast, yolk vitronectin was bound to neither collagen nor gelatin. Neither of the vitronectins bound to bovine serum albumin, which was used as a negative control protein (data not shown).

Heparin binding activity was assayed after treatment of vitronectin with 8 M urea, since 8 M urea appears to prevent nonspecific aggregation of vitronectin and also strongly activates the heparin binding of pure vitronectin (Hayashi et al., 1985; Barnes et al., 1985) as well as endogenous vitronectin in blood serum (Yatohgo et al., 1988). Fig. 7 shows that pure blood vitronectin was bound to heparin-Sepharose in 8 M urea, 0.13 M NaCl, 5 mM EDTA, and 10 mM sodium phosphate (pH 7.7) and eluted with 0.5 M NaCl. In contrast, pure yolk vitronectin was not bound to heparin-Sepharose. Further, none of the vitronectin in a crude sample of yolk plasma was bound to heparin-Sepharose in the presence of 8 M urea (data not shown). Thus, the yolks are less strongly bound to the heparin-binding site as well as a collagen-binding site.

Carbohydrate of Yolk Vitronectin—Through chemical analysis and examination of the reactivity to several kinds of horseradish peroxidase lectins, we previously showed that chicken blood vitronectin contains both O- and N-linked saccharides with sialic acids (Kitagaki-Ogawa et al., 1990). In agreement with our previous results, chicken blood vitronectin of 70 and 65 kDa reacted with ConA, WGA, Allo A, UEA-1, PHA-L, and PNA, but not with LCA (Fig. 8, lane B). Yolk vitronectins of 54 and 45 kDa were stained similarly to blood vitronectin (Fig. 8 lane Y). PNA reacted more strongly with...
both bands of both blood and yolk vitronectins after treatment with neuraminidase, suggesting the existence of terminal sialic acids in at least some of their O-linked saccharides. None of the small polypeptides contaminating yolk vitronectin reacted with any of the lectins, suggesting that they have no bound carbohydrates. On the basis of the above lectin reactivities and the previous carbohydrate analysis (Kitagaki-Ogawa et al., 1990), the O-linked saccharides of both chicken vitronectins are considered to include a (SAα2-3)Galβ1-3GalNAc-Ser/Thr structure, a PNA receptor (where SA is sialic acid). The N-linked saccharides probably have a core structure of Manβ1-4GlcNAcβ1-4GlcNAc-Asn with many Man and GlcNAc residues, as well as a Fuc1-2Gal1-4GlcNAc sequence.

Quantitatively, ConA, Allo A, and PNA stained yolk vitronectin similarly to blood vitronectin, but WGA stained yolk vitronectin more strongly and UEA-L stained it less strongly than blood vitronectin. These differences suggest that yolk vitronectin contains more NeuNAc, less Fuc, and less terminal Gal than blood vitronectin.

To examine the amount of bound carbohydrates, yolk and blood vitronectins were sequentially deglycosylated: sialic acids by neuraminidase, O-linked asialosaccharides by endo-α-N-acetylgalactosaminidase, and finally N-linked saccharides by glycosidase P according to a procedure for sequential deglycosylation of mammalian and avian blood vitronectins (Nakashima et al., 1992). The mass of the two bands of yolk vitronectin decreased during sequential deglycosylation from 54/45 (Fig. 9, lane 1) to 52/43 (Fig. 9, lane 2), 47/38 (Fig. 9, lane 3), and finally 44/35 kDa (Fig. 9, lane 4). The high molecular mass band in lanes 3, 4, 7, and 8 is endo-α-N-acetylgalactosaminidase. The difference in mass between the two vitronectin bands did not vary, suggesting that the two bands contain the same amount and the same composition of bound carbohydrates. Thus, yolk vitronectin contained an approximately 10-kDa mass of carbohydrate: a 2-kDa mass of sialic acids, a 5-kDa mass of O-linked asialosaccharides, and a 3-kDa mass of N-linked saccharides. Similarly, the molecular mass of the major band of blood vitronectin decreased from 70 (Fig. 9, lane 5) to 70 (Fig. 9, lane 6), 65 (Fig. 9, lane 7), and finally 63 kDa (Fig. 9, lane 8) during sequential deglycosylation. Blood vitronectin thus contained an approximately 7-kDa mass of carbohydrate, a less than 1-kDa mass of sialic acids, a 5-kDa mass of O-linked asialosaccharides, and a 2-kDa mass of N-linked saccharides. These results suggest that yolk vitronectin contains more sialic acids and similar amounts of N- and O-linked saccharides compared with blood vitronectin.

**FIG. 8. Lectin binding of vitronectin.** Blood vitronectin (B) or yolk vitronectin (Y) amounting to 6 μg was subjected to SDS-PAGE and transferred from the gel onto nitrocellulose sheets, and then stained with several kinds of horseradish peroxidase-conjugated lectins as indicated on the top. The amount of horseradish peroxidase lectins was sufficient to strongly stain 6 μg of porcine vitronectin as a positive control. CB, the same gel stained for protein with Coomassie Blue before the transfer. The asialo-type vitronectins (aB, aY) were compared with intact vitronectin for PNA staining. Molecular mass (in kDa) is indicated at the left.

**FIG. 9. Deglycosylation of vitronectin.** Yolk vitronectin (lanes 1–4) and blood vitronectin (lanes 5–8) amounting to 3 μg were deglycosylated and stained for protein with Coomassie Blue. Lanes 1 and 5 are intact yolk vitronectin and blood vitronectin, respectively. Sialic acids (lanes 2 and 6), O-linked asialosaccharides (lanes 3 and 7), and N-linked saccharides (lanes 4 and 8) were sequentially removed with neuraminidase, endo-α-N-acetylgalactosaminidase, and glycopeptidase-F (see “Experimental Procedures” for details). Deglycosylation was confirmed by an increment or complete loss of lectin binding activity. The band of high molecular mass in lanes 3, 4, 7, and 8 is endo-α-N-acetylgalactosaminidase. Molecular mass (in kDa) is indicated at the left.

**FIG. 10. Hypothetical structural models of chick blood and yolk vitronectins.** Chick blood vitronectin predominantly consists of a 70-kDa species with a cell-binding RGD sequence near the NH₂ terminus and with a heparin-binding site near the COOH terminus. Yolk vitronectin consists of both 54- and 45-kDa glycoproteins. The 54-kDa vitronectin shares the same NH₂ terminus as blood vitronectin and contains the RGD sequence but not the heparin-binding site. The 45-kDa vitronectin seems to lack both the RGD sequence and the heparin-binding site. The bound carbohydrates are similar, consisting of O-(small hexagons) and N-linked (large hexagons) saccharide chains with more sialic acid (dots) in yolk 54- and 45-kDa vitronectins than in blood vitronectin.

**DISCUSSION**

This is the first report of the existence and biochemical characterization of a distinct vitronectin molecule in chick egg yolk. The yolk vitronectin is composed of 54- and 45-kDa glycoproteins, incorporating an approximately 10-kDa mass of carbohydrate. Yolk vitronectin has cell-spreadng activity but lacks heparin and collagen binding activity.

Since vitronectin was first isolated from human plasma (Hayman et al., 1983; Barnes and Silhustzer, 1983), almost all structural and functional studies of the vitronectin molecule have been concerned with human plasma vitronectin (for reviews, see Preissner (1991) and Tomasini and Mosher (1990)). Human plasma vitronectin separates into two bands of 75 and 65 kDa in SDS-PAGE under reducing conditions, and it has a heparin-binding site toward the COOH terminus, a collagen-binding site possibly toward the NH₂ terminus, and an RGD-dependent cell-spreadng site near the NH₂ terminus. It contains N- but not O-linked saccharides in an amount of 10% (w/w). Its functions include modulation of the activity of membrane attack complement and hemostatic enzymes as well as promotion of cell spreading. Vitronectins from human placenta (Hayman et al., 1983), HepG2 human hepatoma cells (Barnes and Reing, 1985; Nakashima et al., 1992), human yolk sac carcinoma cells (Cooper and Pera, 1988), and human platelets (Preissner et al., 1989) seem to be similar to plasma vitronectin. Blood plasma vitronectins from
13 other animal species have similar properties, except for some variation in apparent molecular mass, number of bands in SDS-PAGE, and carbohydrate composition (Hayman et al., 1985; Kitagaki-Ogawa et al., 1990; Nakashima et al., 1992).

Therefore, the binding activity to heparin and collagen and the cell-spreading activity are considered to be common properties of the vitronectin molecule. Vitronectin-like proteins have recently been reported to exist in a flowering plant, Physarum, brown algae, and a variety of invertebrates (Sanders et al., 1991; Nakashima et al., 1992; Miyazaki et al., 1992; Wagner et al., 1992).

Among them, the vitronectin-like proteins from Physarum and brown algae have been examined and have heparin binding activity (Miyazaki et al., 1992; Wagner et al., 1992). However, we have found in this study that yolk vitronectin, surprisingly, lacks binding activity to heparin and collagen (Figs. 6 and 7). This makes it unique among the vitronectins so far isolated.

The 54-kDa molecule of yolk vitronectin shares the same NH₂-terminal sequence as the 70-kDa blood vitronectin molecule. The NH₂-terminal sequence of the 45-kDa molecule of yolk vitronectin is possibly homologous with the intramolecular sequence beginning at the 50th amino acid from the NH₂-terminus (Table III). These results suggest that the 45-kDa band is an NH₂-terminally truncated product of the 54-kDa vitronectin molecule. This interpretation is supported by the fact that 45-kDa yolk vitronectin lacks cell-spreading activity (Fig. 5). The site required for cell-spreading activity in all vitronectins sequenced so far (Suzuki et al., 1985; Jenne and Stanley, 1985; Seto et al., 1990; Seiffert et al., 1991) is the NH₂-terminal Arg⁴⁵-Gly⁶⁶-Asp⁶⁷ sequence, which should be located in the missing 49-amino acid segment of 45-kDa yolk vitronectin. Similarity in the carbohydrate compositions of the 54- and 45-kDa bands (Figs. 8 and 9) also supports this interpretation and indicates that the NH₂-terminal 49-amino acid peptide of the 54-kDa molecule does not contain any carbohydrate. The difference of 9 kDa between the 54- and 45-kDa molecules is seemingly larger than would be expected for 49 amino acids, which would correspond to roughly a 6-kDa mass. Vitronecitc molecules, however, are known to behave abnormally in SDS-PAGE (Nakashima et al., 1992), and therefore the sizes of 54 and 45 kDa were possibly overestimated. Thus, the apparent difference of 9 kDa is probably derived only from the NH₂-terminal truncation, not from additional COOH-terminal truncation or from intramolecular deletion in the 54-kDa molecule.

In comparison with the major 70-kDa molecule of blood vitronectin, 54-kDa yolk vitronectin is 16 kDa smaller. Considering the carbohydrate masses of 10 and 7 kDa, the polypeptide masses from 49 amino acids, which would correspond to roughly a 6-kDa carbohydrate mass, and therefore the sizes of 54 and 45 kDa were possibly overestimated. Thus, the apparent difference of 9 kDa is probably derived only from the NH₂-terminal truncation, not from additional COOH-terminal truncation or from intramolecular deletion in the 54-kDa molecule. These interpretations are summarized in a tentative structural model (Fig. 10), which compares yolk vitronectin of 54 and 45 kDa with blood vitronectin of 70 kDa.

Yolk vitronectin is present in the solid LDL fraction at low concentration during the purification procedure. Because purified yolk vitronectin becomes soluble in physiological salt solutions, the insolubility seems to be caused by complex formation through ionic bonding with some components in the LDL fraction. This association may depend on the lack of the heparin-binding domain, a highly charged domain.

The collagen-binding site has been suggested to be located in the NH₂-terminal half of the blood vitronectin molecule (Izumi et al., 1988). The yolk vitronectin 54-kDa molecule seems to span this domain, judging from its molecular mass (Fig. 10). This interpretation seems to be inconsistent with the lack of collagen binding activity in yolk vitronectin, unless there is an inhibitory modification of the yolk vitronectin molecule. Further characterization of the collagen binding property should enable us to elucidate this discrepancy in the future.

Our research on yolk vitronectin was initiated to study the role of vitronectin in the early development of the chick embryo, spurred by the work of Thiery and his colleagues on the role of fibronectin, a similar cell-spreading protein, in early development (Thiery et al., 1985, 1989). In egg-laying species, the developing embryo depends completely on the egg components for its physiological and nutritional requirements. Cell adhesion is important during early development, and the abundance of vitronectin but not fibronectin suggests that yolk vitronectin may serve as a main cell adhesion protein in early embryogenesis. The abundance of 45-kDa adhesion proteins in yolk including 30- and 108-kDa proteins in the newt (Komazaki, 1987) and a 160-kDa protein in the sea urchin (Noll et al., 1985). However, they are not yolk vitronectins. The newt 30-kDa protein was reported to be composed of lipovitellin 2 and phosvitin by Komazaki (1987).

Phosvitin from chick egg yolk purchased commercially was examined and found to have cell-spreading activity. Lysozyme from chick egg white has also been reported to have cell adhesion activity (Satta et al., 1980). Thus, the early development of the chick embryo probably depends on the interplay of several kinds of cell adhesion proteins.

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