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A 110-kDa WGA-binding Glycoprotein Involved in Cell Adhesion Acts as a Receptor for Aggregation Factor in Embryos of the Sea Urchin, *Hemicentrotus pulcherrimus*

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ABSTRACT—Glycoproteins that bind wheat germ agglutinin (WGA-binding glycoproteins) were isolated from a Triton extract of an acetone powder of blastula embryos of the sea urchin, *Hemicentrotus pulcherrimus*. The Triton extract was brought to 30% saturation with ammonium sulfate. The supernatant showed inhibitory activity directed against hemagglutination caused by WGA. The supernatant was applied to a column of WGA-agarose and the bound fraction was eluted with N-acetyl-D-glucosamine. The fraction that bound to WGA-agarose was applied to a column of Sephacryl S-400 after reduction and alkylation. After fractionation on Sephacryl S-400, four WGA-binding glycoproteins were identified with molecular weights of 110-, 70-, 66- and 60-kDa after electrophoresis on sodium dodecyl sulfate polyacrylamide gels and staining with WGA labeled with horseradish peroxidase (HRPO). Staining with HRPO-labeled aggregation factor (AF) showed that, of these proteins, only the 110-kDa glycoprotein bound to AF. Moreover, the trypsin fragments of the 110-kDa glycoprotein inhibited the aggregation of dissociated cells of sea urchin embryos caused by AF. The sugar composition of the 110-kDa glycoprotein indicated that this protein contained high levels of a mannose-type oligosaccharide. These results suggest that the 110-kDa WGA-binding glycoprotein on the cell surface may be involved in cell adhesion as a receptor for AF, to which it binds by sugar-lectin type interactions.

INTRODUCTION

Since the discovery of a Ca^{2+}-dependent cell-aggregation factor in the neural retina of chick embryos [1] and sponge [2], a number of cell adhesion molecules (CAMs) have been isolated from various animal cells and characterized [3]. Recently, Ca^{2+}-dependent CAMs, called cadherins, have been reported as factors associated with morphogenesis in mouse embryos [4]. Ca^{2+}-dependent cell-adhesion mechanisms have been proposed from studies on the sponge and the mouse, while Ca^{2+}-indipendent cell-adhesion mechanisms have been reported in chick embryos [5]. Furthermore, the reactions between sugars and lectins play essential roles in intercellular interactions in several organisms [6].

Sea urchin embryos can be dissociated into constituent cells in Ca^{2+}- and Mg^{2+}-free seawater (CMF-SW) [7] and such cells aggregate and reconstitute normal embryos upon the addition of Ca^{2+} [8]. An aggregation factor complex (AFX), a type of CAM, has been found in the supernatant of dissociated cells, and it has been shown to be a gigantic sugar-protein complex which induces the aggregation of cells in a Ca^{2+}-dependent manner [9].

Recently, a Ca^{2+}-binding protein of 1600-kDa, namely aggregation factor (AF), was isolated as a subunit of AFX [10]. This AF had the ability to induce the aggregation of cells in the same manner

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as did AFX and was bound quantitatively to Ca^{2+}, indicative of electrostatic binding between AF and Ca^{2+} (Ca^{2+} bridges) in cell-to-cell aggregation [10]. However, the primary binding of AF to cells was suggested to involve another mecahnism, distinct from the Ca^{2+} bridges, since I^{125}-labeled AF was bound to cell surfaces in the absence of Ca^{2+} [10]. The activity of AF was inhibited by specific sugars, namely N-acetyl-D-glucosamine (GlcNAc) and mannose [10]. This result demonstrates that AF has lectin activity and recognizes GlcNAc and mannose. In addition, the binding activity of cells to AF was lost as a result of the treatment of cells with trypsin [11]. Therefore, it can be concluded that a glycoprotein functioning as the receptor for AF is localized on the cell surface and that AF is bound to the glycoprotein by a sugar-lectin type of interaction.

Recently, we found that wheat germ agglutinin (WGA), a lectin which recognizes GlcNAc, induced dissociation of the cells of embryos of *H. pulcherrimus* and that this effect was eliminated by the addition of GlcNAc [12]. These results suggested that a WGA-binding glycoprotein on the cell surface is involved in cellular adhesion and is a possible candidate for the receptor for AF in the cells of embryos of *H. pulcherrimus*. Therefore, in the experiments reported in this paper, we isolated and purified four WGA-binding glycoproteins and showed that, among them, the 110-kDa glycoprotein is the receptor for AF on *H. pulcherrimus* embryos. In addition, the amino acid and sugar composition of this 110-kDa glycoprotein was analyzed and the involvement of binding of the sugar-lectin type in intercellular interactions was demonstrated.

MATERIALS AND METHODS

Preparation of sea urchin embryos

The eggs of *Hemicentrotus pulcherrimus,* which were obtained by introduction of 0.5 M KCl into the coelom, were cultured after fertilization at 20°C in normal seawater (NSW), at a concentration of 6×10^6 eggs per liter, with gentle agitation. At the hatched-blastula stage, the swimming embryos were immobilized, made to settle at the bottom of the vessel by chilling in an ice bath and collected.

Preparation of dissociated cells and aggregation factor

The concentrated suspension of embryos was washed twice with 5 volumes of cold Ca^{2+}- and Mg^{2+}-free seawater (CMF-SW) by centrifugation at $500 \times g$ for 2 min. Then the embryos were suspended in about 200 volumes of CMF-SW supplemented with penicillin (100 IU/ml), purchased from Meiji Seika (Tokyo, Japan), and incubated with gentle stirring at room temperature for 60 min, until the cells of the embryos were dissociated. The dissociated cells were collected by centrifugation at $1,700 \times g$ for 2 min and used for bioassays of the ability of cell-surface components to aggregate the cells. The cell-free supernatant was used as starting material for purification of AFX. The purification of AF from AFX was carried out as previously reported [10].

Isolation and purification of WGA-binding glycoproteins from dissociated cells

Dissociated cells were washed three times with cold CMF-SW and were defatted by extraction seven times with acetone (5 ml acetone/g wet weight of cells). The defatted precipitate was collected by centrifugation at $1700 \times g$ for 15 min and the final pellet was dried *in vacuo*. The acetone powder was used as the starting material for further purification of WGA-binding glycoproteins.

One gram of the acetone powder was homogenized with a teflon homogenizer in 50 ml of 10 mM Tris-HCl buffer, pH 8.0, that contained 1 M KCl, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% Triton X-100 (extraction buffer) and the mixture was stirred for 24 hr at 4°C. After the extraction, the sample was centrifuged at $15,000 \times g$ for 20 min. The supernatant was collected and dialyzed against 10 mM Tris-HCl buffer, pH 8.0, that contained 10 mM NaCl and 0.01% Triton X-100.

The dialyzed sample was brought to 30% saturation with ammonium sulfate and stirred for 12 hr at 4°C. Then it was centrifuged at $15,000 \times g$ for 20 min. The supernatant (30 sup) was dialyzed

against 10 mM phosphate buffer, pH 7.2, that contained 0.15 M NaCl and 0.01% Triton X-100 and applied to a column of WGA-agarose (2.5 cm i.d. ×2.5 cm), purchased from Seikagaku Kogyo (Tokyo, Japan), which had previously been equilbrated with the same buffer. The bound material was eluted with 0.5 M GlcNAc in the same buffer and the eluate was monitored by measurements of absorbance at 595 nm of samples subjected to staining with Coomassie brilliant blue [13]. The unbound fraction (WGA-0) and the bound fraction (WGA-1) were collected, concentrated by ultrafiltration with a YM-10 membrane (Amicon Corp., Lexington, MA), and the biological activities were assayed by the method described below.

The bound fraction (WGA-1) was dialyzed against distilled water to remove Triton X-100 and then lyophilized. The lyophilized sample, equivalent to 5 mg protein, was solubilized in 1 ml of 1 M Tris-HCl buffer, pH 8.5, that contained 6 M guanidine-HCl and 2 mM ethylenediaminetetraacetic acid (EDTA). Seven mg of dithiothreitol (DTT) were added to the solution to break disulfide bonds and the solution was then kept for 4 hr at room temperature. An aliquot of 17 μl of 4-vinylpyridine was added into the reaction mixture which was then incubated for 4 hr at room temperature.

The reduced and alkylated sample was dialyzed against distilled water to remove the various reagents and lyophilized. The lyophilized sample was solubilized in 1 ml of 0.2 M Tris-HCl buffer, pH 8.5, that contained 6 M guanidine-HCl and 2 mM EDTA, and then it was applied to a column of Sephacryl S-400 (1.0 cm i.d. × 100 cm), purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), which had been equilibrated with the same buffer. The column was eluted with the same buffer at a flow rate of 0.5 ml/min at room temperature. The eluate was monitored by measurements of absorbance at 280 nm. Each fraction was concentrated by ultrafiltration on a YM-10 membrane and dialyzed first against distilled water and then against 5 mM phosphate buffer, pH 6.8, that contained 10 mM NaCl and 0.01% Triton X-100.

Assay of the inhibition of hemagglutination by WGA

WGA-binding activities of glycoproteins were assayed by monitoring the inhibition of hemagglutination by WGA, according to the modified methods of Lis *et al.* [14, 15]. Hemagglutination activity of WGA was first assayed by serial, two-fold dilutions in microtiter U-plates, in order to determine the minimum concentration of WGA for hemagglutination. For assay of hemagglutinating activity, 25 μl of 0.01 M phosphate-buffered saline, pH 7.2, containing 0.8% NaCl and 0.02% KCl (PBS) and 25 μl of a 2% suspension (v/v) of trypsin-treated and formalin-fixed human type 0 erythrocytes in PBS, were added to 25 μl of aliquots of serial, two-fold dilutions of a solution of WGA in PBS. The plates were kept at room temperature for 1 hr and then the hemagglutinating activities were examined. Subsequently, the inhibition assay was carried out as follows. Twenty-five μl of PBS, containing the minimum concentration of WGA necessary for hemagglutination, were added to aliquots of 25 μl of serial, two-fold dilutions of a solution of WGA-binding glycoprotein in PBS, and 25 μl of human type 0 erythrocytes in suspension in PBS were added after a further 30 min. The mixture was kept at room temperature for 1 hr and then the hemagglutinating activity was examined.

One unit of inhibitory activity of WGA-binding glycoproteins in the hemagglutination assay was defined as the amount of protein per one ml in the sample that gave complete inhibition of hemagglutination.

Gel electrophoresis

Electrophoresis on 8% polyacrylamide gels that contained sodium dodecyl sulfate (SDS) was carried out by the method of Laemmli [16]. The gels were stained with silver [17]. A molecular marker kit containing fragments of cytochrome c, purchased from Oriental Yeast (Tokyo, Japan), was used in order for estimations of molecular weights.

Western blotting of WGA-binding glycoproteins and treatment wih HRPO-labelled WGA

WGA labelled with horseradish peroxidase

(HRPO) was prepared by the modified method of Nakane *et al.* [18]. Two mg of HRPO (type VI, 5,000 units), purchased from Sigma Chemical Co. (St. Louis, MO, USA), were reacted with 2 mg of WGA. The proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose sheets for Western blotting [19]. Nonspecific binding was blocked by incubating the sheets with 0.01 M phosphate buffer, pH 7.2, that contained 3% bovine serum albumin (BSA), 0.05% Tween-20 and 0.15 M NaCl (blocking buffer) for 1 hr at room temperature. The sheets were then incubated with the same buffer supplemented with 0.1 mg/ml HRPO-WGA as probe for 1 hr at 4°C. A control experiment was perfomred by treatment of the sheets with a solution of HRPO-WGA that contained 0.5 M GlcNAc. After three washes with 10 mM Tris-HCl buffer, pH 7.2, that contained 0.05% Tween-20 and 0.15 M NaCl, the bound HRPO-WGA on the sheets was visualized by development of color in the enzymatic reaction with 0.02% diaminobenzidine (DAB) and 0.01% H_2O_2 as substrates [20].

Western blotting of WGA-binding glycoproteins and treatment with HRPO-labelled aggregation factor

Preparation of HRPO-labeled aggregation factor, SDS-PAGE and Western blotting were carried out as described above. After the transfer of proteins to nitrocellulose sheets, the sheets were blocked by incubation with 10 mM Tris-HCl buffer, pH 8.0, that contained 3% BSA, 0.05% Tween-20 and 0.15 M NaCl (blocking buffer) for 1 hr at room temperature. The sheets was then incubated with the same buffer supplemented with 0.1 mg/ml HRPO-AF as probe, for 1 hr at 4°C. After three washes with 10 mM Tris-HCl buffer, pH 7.2, that contained 0.05% Tween-20 and 0.15 M NaCl, the bound HRPO-AF on the sheets was visualized as described above.

Treatment of WGA-binding glycoproteins with trypsin

The purified WGA-binding glycoproteins were treated with trypsin to solubilize them in NSW in the absence of Triton X-100. The samples were dialyzed against distilled water for 48 hr to remove Triton X-100 and then lyophilized. Two mg of each lyophilized sample were suspended in 2 ml of PBS, pH 7.2, that contained 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and incubated with 1 mg trypsin (10,000 IU), purchased from Mochida Seiyaku (Tokyo, Japan), for 7 hr at 37°C. To terminate the reaction, 0.075 mg of PMSF was added into the mixture which was then boiled for 10 min. The resultant fragments of glycoproteins were precipitated by the addition of ten volumes of acetone and collected by centrifugation at $1,700 \times g$ for 10 min. Acetone was removed *in vacuo* and the trypsin fragments of glycoproteins were dissolved in 400 μl of CMF-SW for an examination of their biological effects on sea urchin cells.

Effects of WGA-binding glycoproteins on the aggregation of cells caused by AF

Effects of trypsin-treated WGA-binding glycoproteins on the aggregation of cells were assayed in 24-well culture plates, purchased from Falcon (Lincoln Park, New Jersey, USA), as described below. Dissociated cells, prepared as described above, were resuspended in CMF-SW adjusted to pH 8.0 with $NaHCO_3$ and filtered through two sheets of nylon mesh (380-mesh). The population of cells in suspension was counted in a haemocytometer and adjusted to about 5×10^6 cells/ml.

One hundred μl of the minimum concentration of AF in CMF-SW necessary for aggregation of cells, which was determined previously, were added to 100-μl aliquots of serial, two-fold dilutions of the trypsin-treated WGA-binding glycoproteins in CMF-SW, to which 300 μl of Millipore-filtered seawater (MFSW; 0.45 μm pore size) and 100 μl of the suspension of cells in CMF-SW were finally added after shaking for 30 min. The plate was set on a gyratory shaker and rotated at 80 rpm. After incubation at 4°C for 60 min, aggregation of cells was examined under the light microscope and recorded photographically.

Chemical analyses

Protein was estimated by the method of Lowry *et al.* [21] with BSA as a standard. Neutral sugars were measured by the phenol-sulfuric acid method [22] with glucose as a standard. Sialic acid was determined by the resorcin-Cu^{2+}-HCl method [23]

with N-acetylneuraminic acid (NANA) as a standard.

Amino acid analysis was carried out with a reaction-liquid chromatography system, (Hitachi 655, Tokyo, Japan), after hydrolysis of samples with 6 N HCl at 110°C for 24 hr [24].

The composition of sugars was analyzed by gas-liquid chromatography (GLC) of alditol acetates derived from sugars by hydrolysis of samples with 4 N trifluoroacetic acid (TFA) at 121°C for 1 hr [25]. A gas chromatograph, model GC-6A from Shimadzu (Tokyo, Japan), was used and was equipped with a 3% ECNSS-M column (0.3 cm i.d. × 200 cm) at 190°C, for analysis of neutral sugars, and a 3% OV-17 column (0.3 cm i.d. × 100 cm) programmed from 150°C to 205°C (2°C/min), for analysis of amino-sugars, as described previously [26]. Individual derivatives of hexoses and hexosamines were quantitated with a Hitachi data processor 833.

Chemicals

Triton X-100, DAB and GlcNAc were purchased from Wako Pure Chemicals (Osaka, Japan). α-metheyl-D-mannoside was purchased from Fluka AG. (Buchs, Switzerland). WGA was purchased from E. Y. Laboratories, Inc. (San Mateo, CA, USA). Tween-20 was purchased from Bio-Rad Laboratories (Richmonds, CA, USA). PMSF and BSA were purchased from Signma Chemical Co. (St. Louis, MO, USA). All other reagents were of either HPLC or analytical grade.

RESULTS

Purification of WGA-binding glycoprotein

The extract prepared from the acetone powder with the extraction buffer that contained Triton X-100 exhibited inhibitory activity directed against hemagglutination by WGA, as shown in Table 1. However, the extract prepared from the acetone powder with the extraction buffer without Triton X-100 exhibited no such inhbitory activity.

The extract with Triton X-100 was brought to 30% saturation with ammonium sulfate and centrifuged. The supernatant (30 sup) showed the inhibitory activity but the pellet (30 ppt) showed no activity. The supernatant contained 91% of the starting activity and represented a 1.3-fold purification of the activity (Table 1).

The supernatant (30 sup) was applied to a column of WGA-agarose. The elution profile is shown in Figure 1. The activities of the unbound and bound fractions, which designated WGA-0 and WGA-1, respectively, were assayed. Only WGA-1 contained the inhibitory activity. Affinity chromatography allowed recovery of 89% of the activity of the starting material with a 14-fold purification of the activity (Table 1).

We tried to purify WGA-1. However, we were unsuccessful in our attempts to purify intact WGA-binding glycoproteins by chromatography on various gel-filtration columns. Therefore, we tried to fractionate the subunits of WGA-binding glycoproteins after reduction of disulfide bonds. The reduced and alkylated WGA-1 was applied to a column of Sephacryl S-400. Figure 2 shows the elution profile. Only eight tubes, numbered from 59 to 66 (SC-59 through SC-66), contained inhibitory activity (Fig. 2). The gel filtration allowed recovery of 62% of the activity of the starting material. The total amount of the protein in SC-59 through SC-66 corresponded to 2.9% of the protein in the starting material.

TABLE 1. Purificationof WGA-binding glycoprotein

Fraction	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg)	Purification (fold)	Yield of activity (%)
Crude extract	208.9	169.8	0.8	1	100
30% ammonium sulfate 30 sup	153.1	154.6	1.0	1.3	91
WGA-agarose WGA-1	13.6	150.9	11.1	14	89
Sephacryl S-400 SC-60	0.4	7.4	18.5	23	4.4

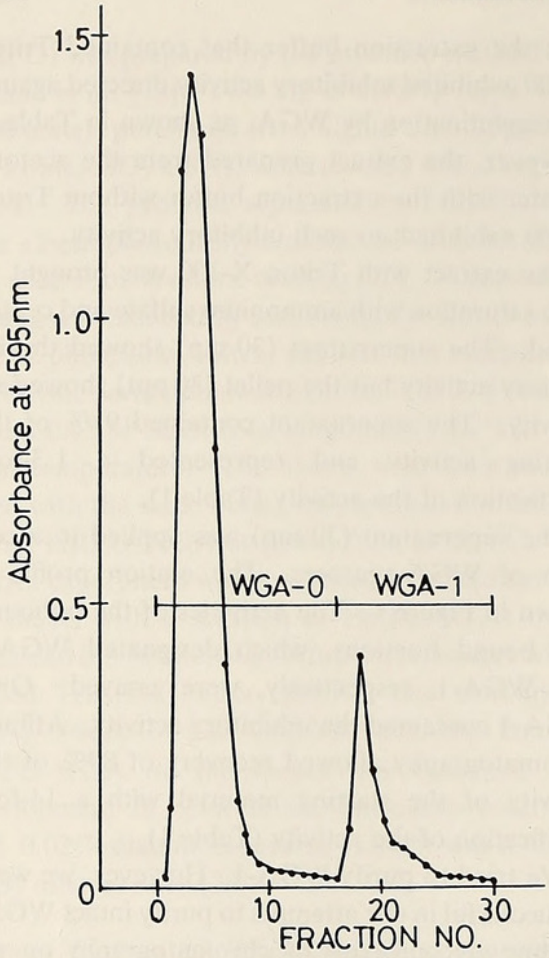

FIG. 1. Profile of the elution from a column of WGA-agarose of the supernatant (30sup) after fractionation with 30% ammonium sulfate. Ten ml of 30 sup, which contained 30 mg protein, were applied to a column of WGA-agarose (2.5 cm i.d. × 2.5 cm) and eluted with 10 mM phosphate buffer, pH 7.2, that contained 0.15 M NaCl, 0.01% Triton X-100 and 0.5 M GlcNAc. Each 10-ml fraction was collected and monitored by measurements of absorbance at 595 nm (●) by the Coomassie-brilliant blue method.

Gel electrophoresis

Figure 3 shows the results of SDS-PAGE of the fractions obtained at each step of the purification. The profile after SDS-PAGE of the 30 sup was nearly as the same as that of crude extract. The profile of WGA-1 gave four major bands that corresponded to molecular weights of 110-, 70-, 66- and 60-kDa and minor bands that corresponded to lower molecular weights. The profile of the pool of fractions 56 through 70 from the chromatography on the column of Sephacryl S-400, after reduction of disulfide bonds, showed that four WGA-binding glycoproteins were separated from other proteins with lower molecular

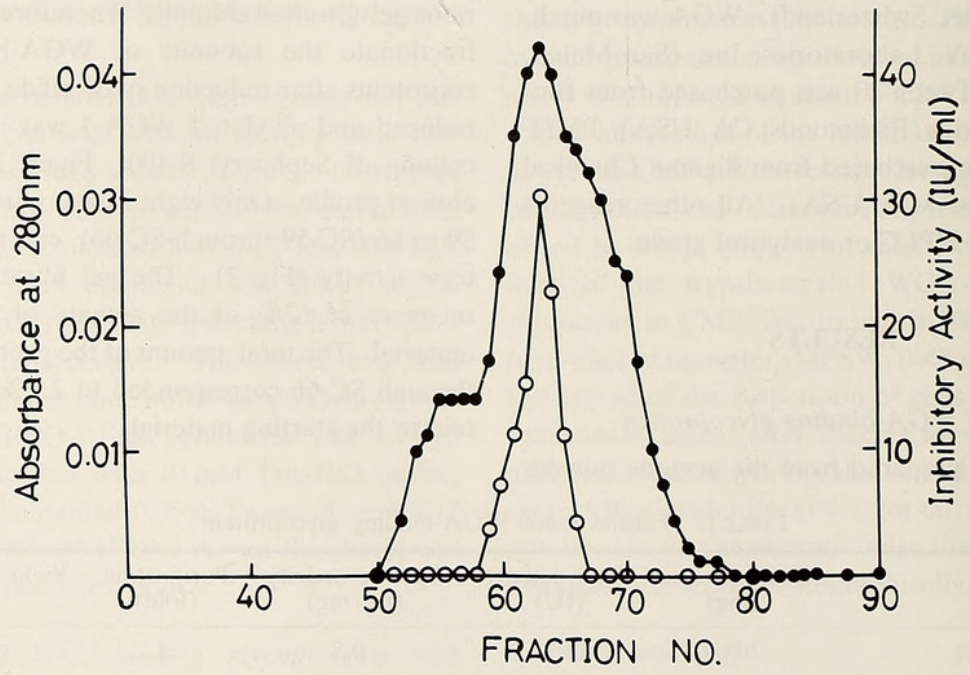

FIG. 2. Profile of the elution from the column of Sephacryl S-400 of WGA-1. WGA-1, equivalent to 5 mg of protein, was reduced and alkylated. The resultant material was dissolved in 1 ml of 0.1 M Tris-HCl buffer, pH 8.5, that contained 6 M guanidine-HCl and 2 mM EDTA, and was applied to the column of Sephacryl S-400 (1.0 cm i.d. × 100 cm). The column was eluted with the same buffer. Each 1-ml fraction was monitored by measurements of the absorbance at 280 nm (●). WGA-binding activity of each fraction was shown by the inhibitory activities of hemaggulutination by WGA (○).

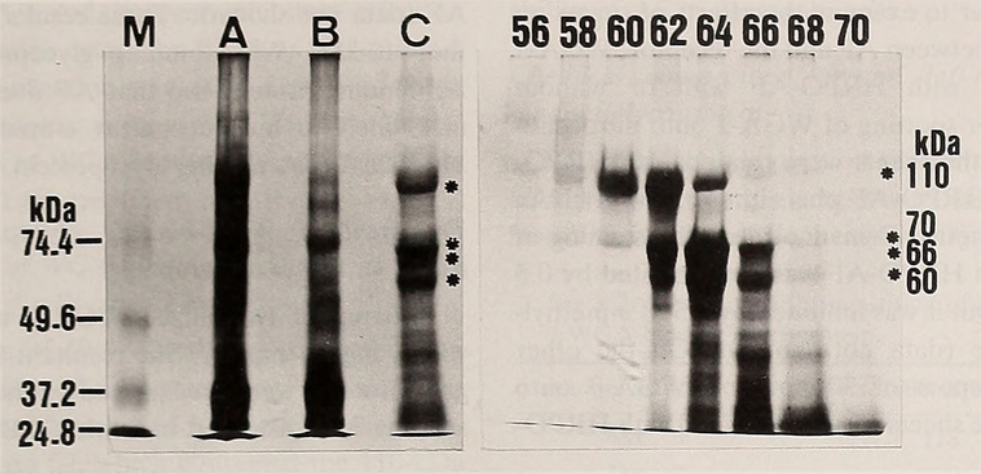

FIG. 3. Results of SDS-PAGE (8% gels) at each step of purification of WGA-binding glycoproteins. Each sample, equivalent to 10–100 μg of protein, was solubilized in sample buffer that contained β-mercaptoethanol and subjected to electrophoresis on an 8% gel. The gels were stained with silver. M, molecular markers; A, crude extract; B, 30% ammonium sulfate supernatant (30 sup); C, the fraction (WGA-1) bound to WGA-agarose; 56~70, fractions eluted from the column of Sephacryl S-400 after reduction of WGA-1.

weights (Fig. 3, lane 56~70) and that a 110-kDa WGA-binding glycoprotein could be isolated (Fig. 3, lane 60).

Western blotting of WGA-binding glycoproteins and treatment with HRPO-WGA

The nitrocellulose sheets onto which the glycoproteins in WGA-1 were transferred from SDS-polyacrylamide gels, after electrophoresis, were treated with HRPO-WGA. In consequence, the major bands which corresponded to molecular weights of 110-, 70-, 66- and 60-kDa were stained with HRPO-WGA and the staining was inhibited by 0.5 M GlcNAc (data not shown). In order to confirm that the 110-, 70-, 66- and 60-kDa glycoproteins were WGA-binding glycoproteins, reduced WGA-1 was loaded again onto a column of WGA-agarose. The bound fraction contained four bands that corresponded to molecular weights of 110-, 70-, 66- and 60-kDa, but the unbound fraction did not generate these bands (data not shown). It was clear, therefore, that the four glycoproteins of 110-, 70-, 66- and 60-kDa bound to WGA-agarose.

Western blotting of WGA-binding glycoproteins and treatment with HRPO-AF

To examine which WGA-binding glycoprotein among the four glycoproteins is the receptor for AF, the 110-, 70-, 66- and 60-kDa glycoproteins were stained with HRPO-AF after SDS-PAGE and Western blotting.

As shown in Figure 4, the band at 110-kDa was stained with HRPO-AF, but the other three bands were not stained. In addition, HRPO-AF that had been previously incubate with 0.5 M GlcNAc retained the ability to bind to the 110-kDa glycopro-

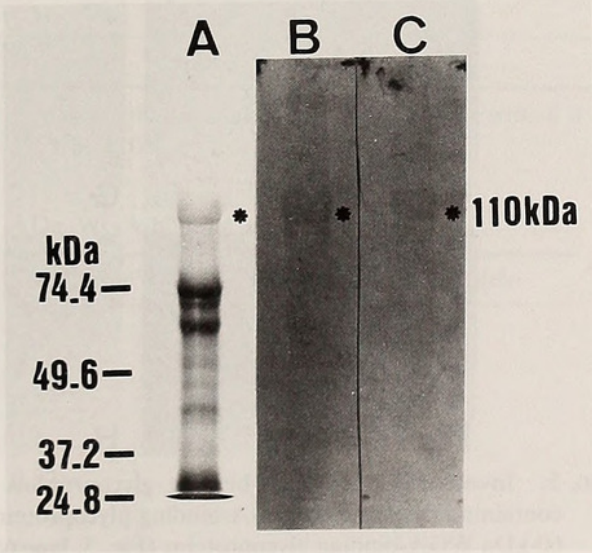

FIG. 4. Staining of WGA-binding glycoproteins with HRPO-AF (see text for abbreviations). The fraction (WGA-1) that bound to WGA-agarose was subjected to SDS-PAGE on 8% gels. Separated proteins were transferred to nitrocellulose sheets. The sheets were then incubated with 0.1 mg/ml HRPO-AF (B) and with HRPO-AF plus 0.5 M GlcNAc (C). A, WGA-1 stained with silver.

tein. In order to examine the effects of sugars on the binding between AF and the receptor, WGA-1 was treated with HRPO-AF with or without sugars. After spotting of WGA-1 onto nitrocellulose sheets, the sheets were treated with HRPO-AF or with HRPO-AF plus either 0.5 M GlcNAc or 0.5 M α-methyl-D-mannoside. The staining of WGA-1 with HRPO-AF was not inhibited by 0.5 M GlcNAc but it was inhibited by 0.5 M α-methyl-D-mannoside (data not shown). On the other hands, the spots of 30 ppt and WGA-0 onto nitrocellulose sheets were not stained with HRPO-AF (data not shown). These results suggest that the 110-kDa WGA-binding glycoprotein is the AF-binding protein and that AF does not recognize GlcNAc but recognizes α-mannose in the 110-kDa WGA-binding glycoprotein.

Treatment of WGA-binding glycoproteins with trypin

Two mg of lyophilized WGA-1 were treated with 1 mg of trypsin. The resultant fragments of glycoproteins were precipitated by the addition of acetone and dissolved in 2 ml of PBS. Trypsin-

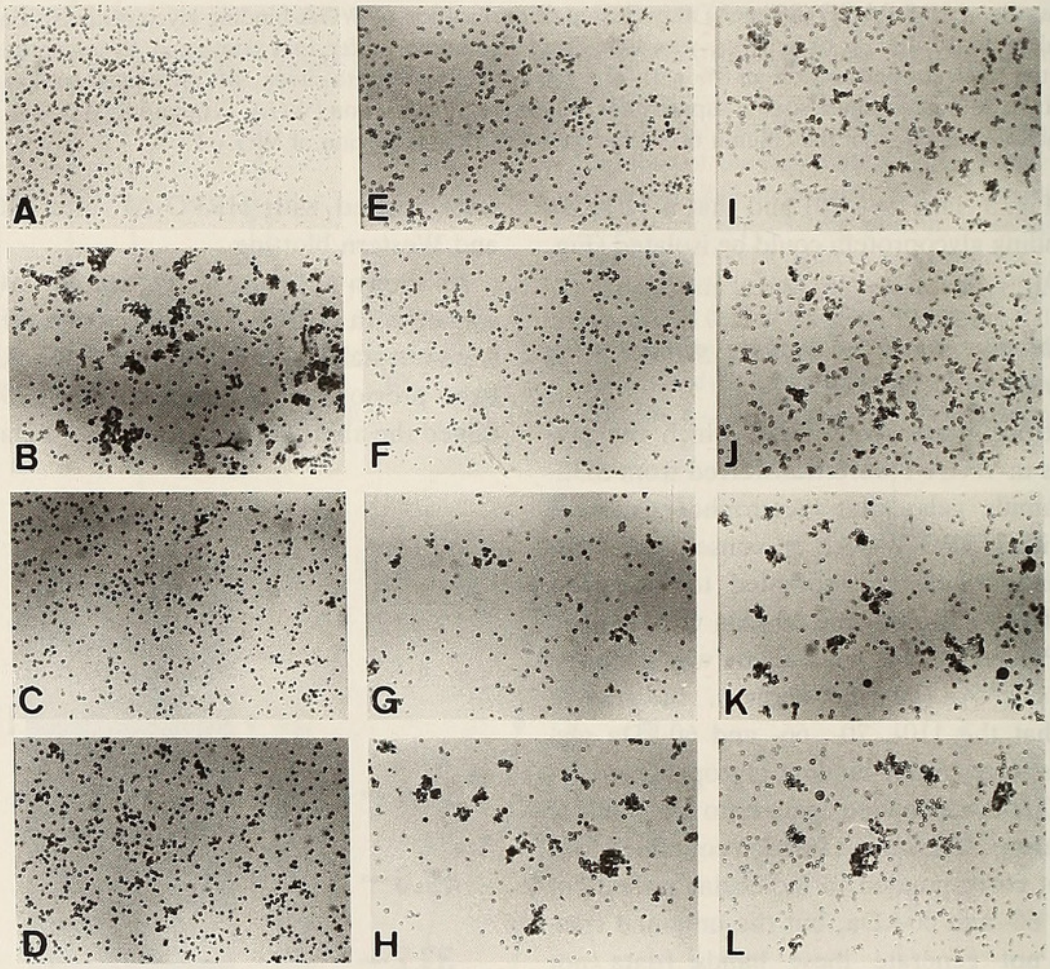

FIG. 5. Involvement of WGA-binding glycoproteins in the aggregation of cells caused by AF. Fraction 60, containing the 110-kDa WGA-binding plycoprotein (Fig. 3, lane 60) and fraction 66, containing the 70-, 66- and 60-kDa WGA-binding glycoproteins (Fig. 3, lane 66) were treated with trypsin to allow them to dissolve in NSW without Triton X-100. The trypsinized fragments of fraction 60 or those of fraction 66 were subjected to two-fold serial dilution and added to dissociated cells simultaneously with the minimum concentration of AF necessary for aggregation of cells. A, dissociated cells; B, cells with AF added at a concentration of 3.125×10^{-2} mg/ml; C, cells with the trypsinized fragments of the 110-kDa glycoprotin added at a concentration of 0.5 mg/ml; D, cells with the trypsinized fragments of the 70-, 66- and 60-kDa glycoproteins added at a total concentration of 0.5 mg/ml; E, F, G, H, cells with the 110-kDa glycoprotein added at concentrations of 0.5, 0.125, 3.125×10^{-2}, 7.8×10^{-3} mg/ml, respectively, plus AF; I, J, K, L, cells with the 70-, 66- and 60-kDa glycoproteins added at total concentrations of 0.5, 0.125, 3.125×10^{-2}, 7.8×10^{-3} mg/ml, respectively, plus AF.

treated WGA-1 had the same inhibitory effect on hemagglutination caused by WGA as that of intact WGA-1 solubilized in a solution of 0.01% Triton X-100 at the same concentration. Thus, the inhibitory activities of WGA-binding glycoproteins were retained after the treatment with trypsin.

Involvement of WGA-binding glycoproteins in the aggregation of cells caused by AF

The effects of WGA-binding glycoproteins on the aggregation of cells caused by AF were examined. Either trypsinized fragments of the materials in fraction 60, which contained the 110-kDa WGA-binding glycoprotein, or of that in fraction 66, which contained 70-, 66- and 60-kDa WGA-binding glycoproteins, were added to dissociated cells simultaneously with the minimum concentration of AF that caused the aggregation of cells. The results are shown in Figure 5. AF had the ability to induce the aggregation of cells, but neither the trypsinized fragments of fraction 60 nor those of fraction 66 had any such ability. However, when the trypsinized fragments of fraction 60 at a concentration of more than 0.125 mg/ml were added to the dissociated cells with AF, the aggregation of the cells was inhibited, while the trypsinized fragments of fraction 66 did not inhibit the aggregation of cells caused by AF even at the high concentration of 0.5 mg/ml. These results suggest that the trypsinized fragments of the 110-kDa WGA-binding glycoprotein neutralized the ability of AF to aggregate cells.

Chemical composition of the 110-kDa WGA-binding glycoprotein

The chemical composition of the purified 110-kDa WGA-binding glycoprotein, in fraction 60 from the column of Sephacryl S-400 (Fig. 3, lane

TABLE 2. Amino acid composition of the 110-kDa WGA-binding glycoprotein

Amino acid	number/1000 amino acids[a]
Asx	128
Thr	79
Ser	82
Glx	100
Pro	39
Gly	68
Ala	58
Val	64
Met	36
Ile	61
Leu	81
Tyr	32
Phe	55
Lys	59
His	16
Arg	42
Total	1000

[a] Values obtained after 24-hr hydrolysis with 6 N HCl at 110°C.

TABLE 3. Sugar composition of the 110-kDa WGA-binding glycoprotein

Sugar	mole %	Residues/molecule[c]	μg/1000 μg of protein
Neutral sugars[a]			
Glucose	1.4	0.18	2.6
Galactose	2.0	0.26	3.6
Mannose	79.0	10.4	144.2
Fucose	2.5	0.32	4.2
Amino sugars[a]			
GlcNAc	15.2	2.0	34.0
GalNAc	0	0	0
Sialic acid[b]	3.3	0.44	10.4
Total	103.4	13.6	199.0

[a] Values obtained by GLC analysis of hydrolyzates prepared by treatment with 4 N TFA at 121°C for 1 hr.
[b] Estimated by the resorcin-Cu^{2+}-HCl method.
[c] Values calculated from mole % taking the value for GlcNAc as 2.0.

60), was examined. The amino acid and sugar compositions are shown in Tables 2 and 3, respectively. As shown in Table 2, the 110-kDa glycoprotein was rich in Asx and Glx, a standard feature of glycoprotein. As shown in Table 3, the 110-kDa glycoprotein was composed of about 20% sugars. The major sugar was mannose which was present in a molar ratio of 10.4 : 2.0 with respect to GlcNAc, indicating the presence of typical high-mannose type sugar chains which are usually composed of 8 mannose residues and 2 GlcNAc residues [27, 28]. The levels of gluocose, galactose, fucose were very low, and GalNAc was not detectable.

DISCUSSION

The extract prepared from the acetone powder with the extraction buffer without Triton X-100 exhibited no inhibitory activity against hemagglutination by WGA. This result suggests that the WGA-binding glycoprotein is a hydrophobic protein in cell membranes.

When reduced WGA-1 was incubated with HRPO-WGA after the Western blotting and SDS-PAGE, bands that corresponded to molecular weights of 110-, 70-, 66- and 60-kDa were stained (data not shown). Furthermore, when reduced WGA-1 was applied to a column of WGA-agarose, only four glycoproteins of 110-, 70-, 66- and 60-kDa were bound to the affinity column. These results demonstrate that the 110-, 70-, 66- and 60-kDa glycoproteins are WGA-binding glycoproteins. However, unless WGA-1 was reduced, WGA-1 did not enter in 8% SDS-polyacrylamide gel. Moreover, when intact WGA-1 was applied to the column of Sephacryl S-400, WGA-1 passed through the gel. These results suggest that WGA-1 is a very large molecule that is composed of subunits. Therefore, it is probalbe that the four WGA-binding plycoproteins are asociated with one anothe through disulfide bonds.

We demonstrated previously that addition of WGA to the culture medium of embryos caused the dissociation of the embryos [12]. In addition, the aggregation of cells caused by AF fails to occur after treatment of the surface of cells with trypsin [11]. These results suggest that WGA-binding glycoproteins in cell membranes are involved in the adhesion of cells in sea urchin embryos. Therefore, we examined the effects of WGA-binding glycoproteins *in vivo*. The trypsin-treated WGA-1 also inhibited the aggregation of cells caused by AF (data not shown). These result suggests that WGA-binding glycoproteins are involved in cell adhesion as receptors for AF. It is probable that dissociation of embryos by WGA is due to the inhibition of binding between AF and the receptors for AF.

I^{125}-labeled AF binds to the surface of cells in the absence of Ca^{2+} [10], and so the primary binding of AF to cells was thought to involve a mechanism distinct from the Ca^{2+} bridge. Furthermore, the activity of AF is inhibited sugar-specifically by GlcNAc and mannose [10], and erythrocytes after preincubation with AF are not aggregated by WGA [10]. These results demonstrate that AF has lectin-like activity and recognizes GlcNAc and mannose. In addition, since the binding of AF to cells is lost after treatment of cells with trypsin [11], it seems likely that receptors for AF are glycoproteins exposed to the cell surfacce. From these results, it is strongly suggested that AF binds to receptors on the cell surface by a sugar-lectin type of binding. When WGA-1, containing the 110-, 70-, 66- and 60-kDa glycoproteins was incubated with HRPO-AF, only band at 110-kDa was stained with HRPO-AF, and the other three bands were not stained. The binding between the 110-kDa glycoprotein and HRPO-AF was not inhibited by 0.5 M GlcNAc but was inhibited by 0.5 M α-methyl-D-mannoside. From these results, it appears that the 110-kDa WGA-binding glycoprotein is an AF-binding protein and that AF does not recognize GlcNAc but recognizes α-mannose in the 110-kDa WGA-binding glycoprotein. When we used a ConA-sepharose column to purify AF-binding protein, we could not obtain the active fraction by the elution with 0.5 M α-methyl-D-mannoside. Therefore, we used WGA-agarose instead of ConA-sepharose to purify AF-binding protein, even if AF may recognize α-mannose.

In our experiments *in vivo*, when either the trypsinized fragments of the 110-kDa glycoprotein or those of the other three glycoproteins were added to dissociated embryonic cells simula-

taneously with AF, only the trypsinized fragments of the 110-kDa glycoprotein were found to inhibit the aggregation of cells caused by AF. These results support our conclusion that 110-kDa WGA-binding glycoprotein is at least one of the receptors for AF.

Sugar analysis of the purified 110-kDa WGA-binding glycoprotein demonstrated that it contained 10.4 residues of mannose and 2.0 residues of GlcNAc per molecule. The constitution of the oligosaccharide in this glycoprotein is indicative of a typical high-mannose type of sugar chain, which is usually composed of 8 residues of mannose and 2 residues of GlcNAc [27, 28]. The results are consistent with the observation that the band at 110-kDa was stained with HRPO-ConA, which recognized α-mannose (data not shown). Furthermore, the staining of the 110-kDa band with HRPO-AF was inhibited not by GlcNAc but by α-methyl-D-mannoside. From these observations, it is strongly suggested that AF recognizes α-mannosyl residues on the 110-kDa glycoprotein which is exposed on the surface of sea urchin cells and that binding between AF and the 110-kDa glycoprotein involves interactions of the sugar-lectin type.

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