Purification and Characterization of an 82-kD Membrane Protein As a Neurite Outgrowth Factor Binding Protein: Possible Involvement of NOF Binding Protein in Axonal Outgrowth in Developing Retina

Hideo Taniura, Che-Hui Kuo,* Yokichi Hayashi,* and Naomasa Miki

Department of Pharmacology I, Osaka University School of Medicine, Osaka 530, Japan; and *Department of Pharmacology, Cancer Research Institute, Kanazawa University, Kanazawa 920, Japan

Abstract. Neurite outgrowth factor (NOF) is a glycoprotein isolated from an extract of gizzard that induces neurite outgrowth from cultured retinal or ciliary ganglionic (CG) neurons. We have reported that a glycoprotein of ~82 kD solubilized from gizzard muscles binds to NOF (ligand blotting) and inhibits the neurite promoting activity of NOF (inhibition assay). The 82-kD protein (NOF binding protein) was purified from gizzard muscle membranes as a doublet band on SDS-PAGE and a polyclonal antibody was raised against it. An NOF binding protein in developing retina exhibited the same physicochemical properties as that of the gizzard muscle. Quantitative decrease in NOF binding protein in embryonic retinas was observed after day 11 by the inhibition assay, ligand blotting, and immunoblotting, its decrease being parallel with reduction of NOF-induced neurite outgrowth of embryonic retinas. In an immunohistochemical study, the antibody stained only the optic fiber layers of the retinas of 8-d embryos, and this staining was no longer detectable in retinas of 18-d embryos. These results suggest that the 82-kD protein is a novel membrane protein that behaves as an NOF receptor and that the loss of neuritic response of the retinal neurons to NOF reflects a decrease in NOF receptor molecules.

CULTURED neurons from the peripheral or central nervous system can extend neurites along selected substrata with which their advancing growth cones have high affinity. Neurite extension is known to be regulated by several factors, such as extracellular substances, the developmental stage of neurons and cell–cell interactions. Recent evidence suggests that laminin or laminin-like molecules may play a crucial role in developing (14, 15) and regenerating (9, 16) nervous tissues. We have purified a neurite outgrowth factor (NOF;[1] ~700 kD) from an extract of chicken gizzard that promotes neurite outgrowth from cultured ciliary ganglionic (CG) and retinal neurons, and reported that NOF is an extracellular matrix protein belonging to the laminin family, although it differs from laminin in its subunit structure and antigenicity (6). Integrins are known to serve as receptors for a variety of extracellular matrix proteins (22). They are a large family of glycoprotein complexes consisting of heterodimers with subunits of 120–200 kD. Integrins recognize the Arg-Gly-Asp (R-G-D) sequence, which is included in the cell binding domain of extracellular matrix molecules (21). To determine whether neurite outgrowth induced by NOF is mediated by a specific NOF receptor or integrins, we attempted to isolate NOF-receptor molecules on the assumption that if a tissue extract contains NOF-receptor, it should bind to NOF (ligand blotting), and also inhibit the neurite promoting activity of NOF (inhibition assay). We have reported that NOF binding protein identified in this way is present in chick gizzard muscles and that an 82-kD glycoprotein solubilized from chicken gizzard muscles is a possible NOF receptor (19). Neurite extension from CG or retinal neurons induced by NOF depends strictly on the developmental stage (8, 11). The neuritic response of these neurons to NOF is maximal in 6–10-d embryos and thereafter decreases gradually. In the present paper, we report the purification and properties of an NOF-binding protein (82-kD protein) from gizzard muscle membranes and demonstrate that an NOF binding protein in developing retinas exhibits the same properties as that of the gizzard muscles. We also report studies on whether quantitative change on NOF-binding protein in the developing retina is correlated with loss of neuritic responsiveness to NOF.

1. *Abbreviations used in this paper*: CG, ciliary ganglionic; NOF, neurite outgrowth factor; PBS(-), PBS without Ca^{++} and Mg^{++}; PORN, poly-DL-ornithine.

Materials and Methods

Chemicals

Dulbecco's PBS without Ca^{++} and Mg^{++} (PBS [−]), FCS, DME and a mixture of penicillin and streptomycin were purchased from Gibco Labora-

tories (Grand Island, NY). Sepharose CL-6B, Sephacryl S-300 and protein A-Sepharose were from Pharmacia Fine Chemicals (Piscataway, NJ). Molecular standards for SDS-PAGE and for gel filtration, a biotin blotting kit for detection of molecular weight markers and horseradish peroxidase conjugated to anti-rabbit and anti-mouse IgG were from Bio-Rad Laboratories (Cambridge, MA). DEAE-cellulose (DE 52) was from Whatman Inc. (Clifton, NJ). AF-red Toyopearl was from Toyo soda. Fluorescent-labeled antibody to rabbit IgG was from Vector Laboratories (Burlingame, CA). NP-40 and Coomassie brilliant blue were from Nakarai. Laminin, Triton X-114, and poly-DL-ornithine (PORN) were from Sigma Chemical Co. (St. Louis, MO). BCA protein assay reagent for the detection of protein concentration in detergent solution was from Pierce. Other compounds were of reagent grade.

Cultures of CG and Retinal Neurons

Before culture of CG or retinal neurons, the wells of culture plates were coated with PORN-wells. CG from 8-d-old chick embryos were removed and separated into single cell suspensions as previously described (18). Briefly, excised CG were incubated for 20 min at 37°C in PBS(−) containing 0.05% trypsin. They were then washed twice with DME supplemented with 10% FCS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin, and a single cell suspension was obtained by repeated in a Pasteur pipette. The volume of cell suspension was then adjusted to 1 ml of medium/2 ganglia (~14,000 cells/ml), and a volume of 40 μl of cell suspension was introduced into 96-well culture plates. The cells were cultured at 37°C in a humidified atmosphere of 10% CO_2 in air for 5 h. Neuroretinas were removed from 8-d-old chick embryos in PBS(−), cut into 0.5–1-mm portions with scissors and placed in 24-well culture plates.

Bioassay for NOF Activity

Bioassay was carried out as previously described (7). The preparation containing NOF was centrifuged for 30 min at 10,000 *g*, and the supernatant was collected aseptically. An aliquot of the supernatant was mixed with 40 μl of PBS(−) and introduced into PORN-wells with 250 μl of sterile distilled water. After incubation for 24 h at 37°C, the solutions were removed from the wells (PORN-wells precoated with NOF). The plates were then washed with distilled water and 40 μl of PBS(−) was added to the PORN-wells precoated with NOF and cultured at 37°C for 5 h. The NOF activity in each well was determined as a percentage of neurons possessing neurites among the total surviving neurons (designated as a percentage neurite outgrowth).

Assay of Inhibition for NOF Binding Protein (82-kD Membrane Protein)

Detergent-solubilized materials (30 μl) were diluted to 200 μl with a solution of 20 mM Tris HCl (pH 7.5) and 2 mg/ml of BSA and incubated for 12 h at 37°C in PORN-wells precoated with NOF. CG neurons from 8-d-old chick embryos were washed with distilled water and PBS(−), cultured for 5 h, and then this NOF activity was measured.

Immunoblotting and Ligand Blotting

The methods used for preparations of polyclonal and monoclonal (1-4D) antibodies against NOF were described previously (6, 8). Immunoblotting onto a nitrocellulose sheet was also carried out as described previously (6). Ligand blotting was carried out in the same way as immunoblotting except that, before staining, materials transferred to nitrocellulose sheets were incubated with NOF (~100 μg protein/ml) in PBS(−) containing 3% BSA and 0.5% NP-40 at 37°C for 2 h and washed with 0.05% Tween 20 in PBS(−). A biotin blotting kit was used for staining marker proteins.

Preparation of NOF

NOF was purified from adult chicken gizzard muscles as described previously (6), and the Sepharose CL-6B fraction (~80% purity) was used in this study.

Preparation of Antibody against NOF Binding Protein (82-kD Protein)

Chromatographically purified 82-kD protein was separated by electrophoresis on SDS-gel (7.5%) (13), and the 82-kD protein was extracted from the SDS-gel. The acetone-treated sample was suspended in PBS(−) and used for immunization. Antiserum against the 82-kD protein was raised in a rabbit (~2 kg) as described previously for NOF (6). Samples of 20 μg/rabbit of 82-kD protein emulsified in Freund's complete adjuvant were injected 8 times in 4 wk. The IgG fraction was obtained using a protein A-Sepharose affinity column and dialyzed against PBS(−).

Preparation of a Membrane Fraction from Chicken Embryonal Retinas and Triton X-114 Phase Separation

Chicken embryonal retinas (8–18-d-old) were homogenized with 0.32 M sucrose (0.5 ml/retina) in a Polytron homogenizer. The mixture was centrifuged at 1,000 *g* for 10 min. The pellet was removed, the supernatant was centrifuged at 18,000 *g* for 30 min, and the pellet was named the P_2 fraction. Triton X-114 phase separation was carried out essentially by the method of Bordier (1). The P_2 fraction from chicken embryonal retinas was solubilized in 1% Triton X-114, 10 mM Tris HCl (pH 7.5), and 0.15 M NaCl for 60 min at 4°C (0.5 ml/2 retinas), and the resulting supernatant was recentrifuged at 100,000 *g* for 60 min. Samples of 500 μl in 1.5 ml conical microfuge tubes were incubated for 10 min at 32°C and centrifuged for 3 min at 300 *g*. Aliquots of the two phases were precipitated with acetone.

Immunohistochemistry

Indirect immunohistochemical examination of thin sections onto gelatin-coated coverslips cut in a cryostat was performed as described previously (7). Sections were incubated with antiserum against NOF binding protein at a dilution of 1:500 for 12 h at 4°C. After staining with fluorescein-conjugated goat anti-rabbit immunoglobulin at a dilution of 1:500 for 12 h at 4°C, the samples were examined with an Olympus IMT-2 fluorescence microscope.

Results

Purification of NOF Binding Protein from Chicken Gizzard Muscle

The NOF binding protein (82-kD membrane protein) was purified from chicken gizzard muscle as described below. The purification of NOF binding protein by this procedure has been repeated several times with similar results. All purification steps were performed at 0–4°C.

Step 1: Crude NP-40 Extract

About 200 *g* of fresh gizzard muscles were cut into small pieces and mixed with 5 vol of PBS(−). The mixture was homogenized in a Polytron homogenizer operated at maximum speed for 30 s. The homogenate was centrifuged at 13,000 *g* for 30 min, and the pellet was suspended in PBS(−) and stirred overnight. Then it was centrifuged at 13,000 *g* for 30 min, and the pellet was suspended with 5 vol of 10 mM Tris acetate (pH 8.0) and stirred for 2–3 h. The mixture was centrifuged and the pellet was extracted with 5 vol of 10 mM Tris acetate (pH 8.0), 0.5% NP-40 and 2 mM PMSF for 60 min with constant stirring. The mixture was centrifuged for 30 min at 13,000 *g*, and the resulting supernatant was used as crude NP-40 extract.

Step 2: Acetate Treatment

The crude NP-40 extract was adjusted to 0.02 M acetic acid and incubated for 60 min. Insoluble material was removed by centrifugation at 13,000 *g* for 10 min, and the supernatant was adjusted to pH 7.0–8.0 by addition of 1 M Tris base.

Step 3: Acetone Precipitate

The acid-treated sample was concentrated on a Diaflo membrane YM 100 (Amicon Corp., Danvers, MA) and then 6

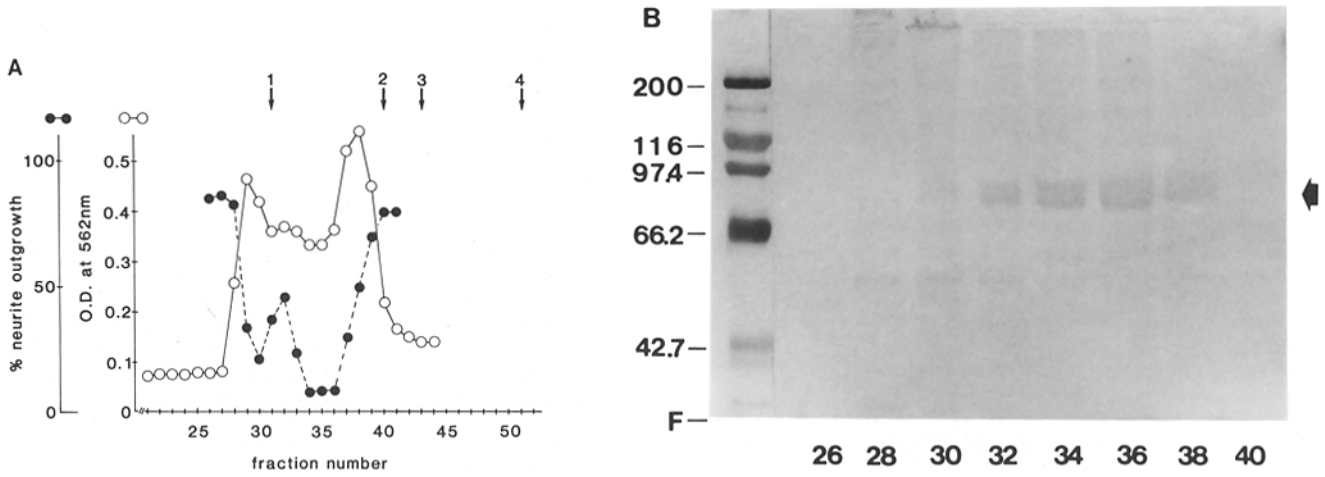

Figure 1. Gel filtration of acetone-precipitated sample on Sephacryl S-300. (*A*) An acetone-precipitated sample (~6.3 mg protein/ml, 5 ml) was applied to a Sephacryl S-300 column (2.6 × 96 cm) and fractions of 8 ml were collected. The protein concentration of each fraction was measured as $A_{562\,nm}$ with BCA protein assay reagent (○) and an aliquot of each fraction was assayed for inhibition of NOF activity (●). Arrows show the positions of marker proteins eluted in the same conditions: (*1*) thyroglobulin (670 kD); (*2*) gamma globulin (158 kD); (*3*) ovalbumin (4.4 kD); (*4*) myoglobin (1.7 kD). (*B*) Ligand (NOF) blot of fractions obtained by Sephacryl S-300 gel filtration. Aliquots of each fraction obtained by Sephacryl S-300 gel filtration were separated on 7.5% polyacrylamide gel under nonreducing conditions, and proteins were transferred electrophoretically to a nitrocellulose sheet. The sheet was incubated with NOF (~100 μg protein/ml) for 2 h at 37°C and then washed with 0.05% Tween 20 in PBS(−). The NOF binding protein was detected with anti-NOF serum. Lane numbers correspond to fraction numbers on Sephacryl S-300 gel filtration. The left lane shows marker proteins detected with a biotin blot kit (Bio-Rad Laboratories). These markers are myosin (200 kD), beta galactosidase (116 kD), phosphorylase b (97.4 kD), BSA (66.2 kD), and ovalbumin (42.7 kD). F means dye front. The figures on the left represent molecular sizes in kilodaltons.

vol excess of acetone were added. The mixture was kept at −20°C overnight and then centrifuged at 6,000 *g* for 15 min. The precipitate was suspended in PBS(−), precipitated by centrifugation at 8,000 *g* for 30 min, and redissolved in 10 mM Tris acetate (pH 8.0), 1 mM EDTA and 0.5% NP-40. The resulting preparation was centrifuged for 90 min at 100,000 *g*, and the supernatant was used for further purification.

Step 4: Gel Filtration on Sephacryl S-300

The acetone-treated sample (~6.3 mg protein/ml, 5 ml) was applied to a Sephacryl S-300 column (2.6 × 96 cm), that had been equilibrated with 10 mM Tris acetate (pH 8.0), 1 mM EDTA and 0.5% NP-40. Elution was carried out with the same buffer at a flow rate of 16 ml/h, and fractions of 8 ml were collected. Inhibition assay (Fig. 1 *A*) and ligand (NOF) blotting (Fig. 1 *B*) showed that NOF binding protein was eluted in fractions Nos. 33–36, so these fractions were combined. The apparent molecular mass of material with activity was in the range of 200–300 kD.

Step 5: AF-red Toyopearl Chromatography

The fractions with activity (33–36) from the Sephacryl S-300 column were combined and applied to an AF-red Toyopearl

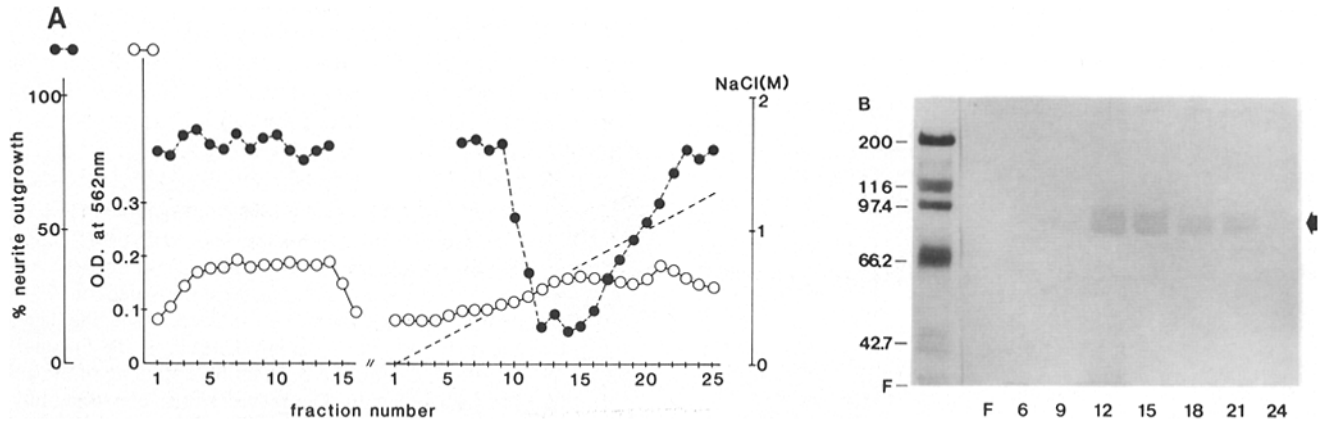

Figure 2. AF-red Toyopearl chromatography of the active fraction obtained by gel filtration. (*A*) The fractions obtained by gel filtration were combined (~30 ml) and applied to an AF-red Toyopearl column (1 × 10 cm). Material was eluted with a gradient of NaCl (0–2 M). Fractions of 4 ml before elution and of 2 ml during elution were collected. Protein concentration (○) and inhibition of NOF activity (●) were measured. (*B*) Ligand (NOF) blot using the fractions eluted from AF-red Toyopearl. The procedures used were as described for Fig. 1. (*B*) Lane numbers correspond to fraction numbers of eluate. Lane *F* is a sample of the flow-through fraction (number 10 before elution). The left lane shows the positions of marker proteins.

Table I. Summary of Purification of NOF Binding Protein

Fraction	Total protein	ID_{50}	Purification	Recovery
	mg	*μg*		
Gizzard muscle	200,000	–	–	–
Crude NP-40 extract	750	3.3	1	100
Acetate treatment	380	1.6	2	105
Acetone precipitate	157.5	0.36	9	193
Sephacryl S-300	37.5	0.12	27	138
AF-red Toyopearl	1	0.011	300	40

ID_{50} values are expressed as protein concentrations causing the half maximal inhibition of NOF (100 ng protein/well) activity.

column (1 × 10 cm) that had been equilibrated with 10 mM Tris acetate (pH 8.0), 1 mM EDTA and 0.5% NP-40. The flow rate was maintained at 0.5 ml/min. The column was washed with the same buffer, and then developed with a linear gradient of increasing NaCl concentration (0–2 M) in 10 mM Tris acetate (pH 8.0), 1 mM EDTA and 0.5% NP-40, and fractions of 2 ml were collected. Inhibition assay (Fig. 2 A) and ligand (NOF) blotting (Fig. 2 *B*) showed that the NOF-binding protein (82-kD protein) was eluted in fractions Nos. 12–16, and so these fractions were combined as purified NOF binding protein.

The purification of NOF binding protein is summarized in Table I. Measurement of the NOF binding protein activity in each step by inhibition assay showed that the overall purification was ~300-fold with a yield of ~40% from the crude NP-40 extract. The recoveries in steps 2 and 3 were >100%, possibly because of the dissociation of NOF from a complex of NOF and NOF binding protein during these steps. The content of NOF binding protein in adult gizzard membranes was estimated to be 0.5–1% by measurement with the ligand and immunoblotting. The NOF-binding protein was stable and could be stored at −80°C for several months without any loss of activity.

Physicochemical Properties of NOF Binding Protein and Its Antibody

On analytical gel electrophoresis in polyacrylamide (7.5%) gel containing SDS (0.1%) under nonreducing conditions, the purified NOF-binding protein (82-kD protein) gave a doublet band, the ratio of the stained bands differing in different samples. A band of ~200 kD was also observed under nonreducing and reducing conditions (Fig. 3, lanes *a* and *b*). This band of higher molecular mass seemed to be an aggregated form of the 82-kD protein and its intensity increased during repeated column chromatographies (ion-exchanger or gel-filtration). Purified NOF binding protein was subjected to electrophoresis on SDS-gels and the 82-kD protein band was extracted from the SDS-gels as an immunogen. The specificity of antiserum against the 82-kD protein was examined by an Ouchterlony double-immunodiffusion test with purified NOF binding protein and a crude acetone-treated sample. Antiserum against the 82-kD protein gave a single precipitation line against the purified NOF binding protein and the crude acetone-treated sample (data not shown). On immunoblot analysis, polyclonal serum against the 82-kD protein reacted strongly with an 82-kD protein band and also reacted with bands of higher molecular mass in nonreducing and reducing conditions (Fig. 3, lanes *e* and *f*). But antiserum against the 82-kD protein reacted only with an 82-kD band in the crude acetone-treated sample, which was identical to that of purified NOF binding protein (Fig. 5, lane *c*). When antiserum against 82-kD protein had been absorbed with 82-kD protein, it no longer reacted with the 82-kD band or those of higher molecular mass on immunoblotting (data not shown). These observations indicated that the bands of ~200 kD were those of dimers or polymers of the 82-kD protein. Under reducing conditions, the NOF binding protein migrated slower as a single 83-kD band with a faint dimer band (Fig. 3, lane *b*), indicating that the NOF binding protein contains S-S bonds. When samples of purified NOF binding protein were used for ligand blotting (Fig. 3, lanes *c* and *d*), a doublet band at 82 kD was clearly detected under nonreducing conditions (Fig. 3, lane *c*), but no bands were detected under reducing conditions (Fig. 3, lane *d*). These findings suggest that the internal disulfide bonds of NOF binding protein are essential for binding of NOF with NOF binding protein. The effects of ionic strength and chelating agents were examined by ligand blotting (Fig. 4). The stained bands were quantified by their intensities of staining for protein at $A_{450\ nm}$. The intensity of the 82-kD

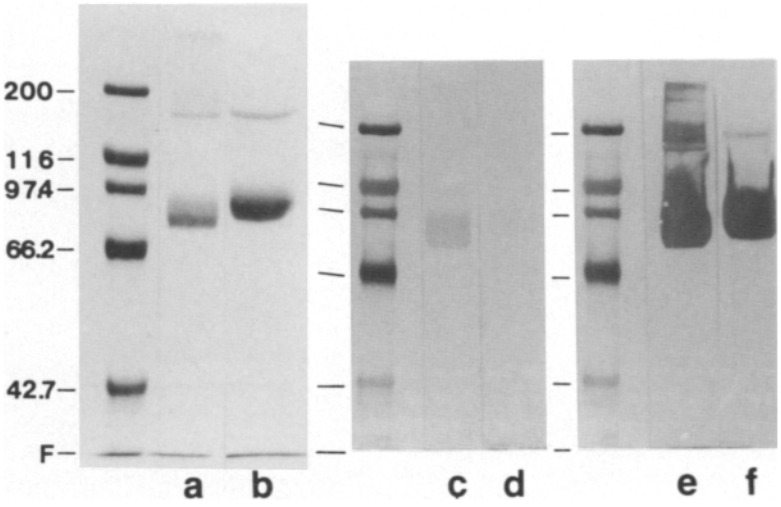

Figure 3. SDS-PAGE of the purified 82-kD protein from gizzard membranes. The purified NOF binding protein fraction (~13 μg protein) from the AF-red Toyopearl column was separated on 7.5% SDS-polyacrylamide gel under nonreducing (lanes *a*, *c*, and *e*) or reducing (lanes *b*, *d*, and *f*) conditions. Coomassie brilliant blue staining of the gel (lanes *a* and *b*). The gel was blotted electrophoretically onto nitrocellulose sheets, and NOF binding ability was detected by ligand blotting (lanes *c* and *d*). Proteins on the blotted sheets were stained with antiserum against NOF binding protein (lanes *e* and *f*). The left lanes of each panel show the positions of marker proteins.

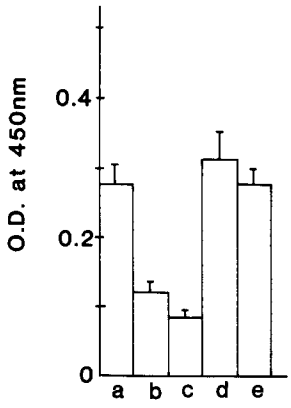

Figure 4. Effects of ionic strength and chelating agents on ligand binding to purified NOF binding protein (ligand blots). The purified NOF binding protein (~10 μg protein) was subjected to electrophoresis on 7.5% SDS-polyacrylamide gel under nonreducing conditions, transferred to nitrocellulose sheets and incubated with NOF in PBS(−), 3% BSA and 0.5% NP-40 (lane *a*), in the presence of 1 M NaCl (lane *b*), 2 M NaCl (lane *c*), 5 mM EGTA (lane *d*), or 5 mM EDTA (lane *e*). NOF binding proteins were detected with anti-NOF serum. The bands were quantified by their color intensity at $A_{450\ nm}$. Columns and bars represent means and SD_S for four independent determinations.

band was reduced by increase in the concentration of NaCl (Fig. 4, lanes *b* and *c*). Integrins need calcium ion for their binding to laminin or fibronectin (22), so the requirement for calcium ions was also examined by ligand blotting. No inhibitory effect of EGTA or EDTA on the association between NOF and NOF binding protein was observed (Fig. 4, lanes *d* and *e*) when nitrocellulose sheets to which NOF binding protein had been transferred were incubated with NOF in PBS(−) containing 3% BSA and 0.5% NP-40 and 5 mM EDTA or EGTA. Similar absence of requirement for calcium was observed with solubilized native protein by dot-blot analysis (data now shown). Treatment of purified NOF binding protein with trypsin (200 μg/ml) for 2 h at 37°C or for 2 min at 100°C abolished its inhibitory activity (data not shown), consistent with previous results with a crude NP-40 extract (19).

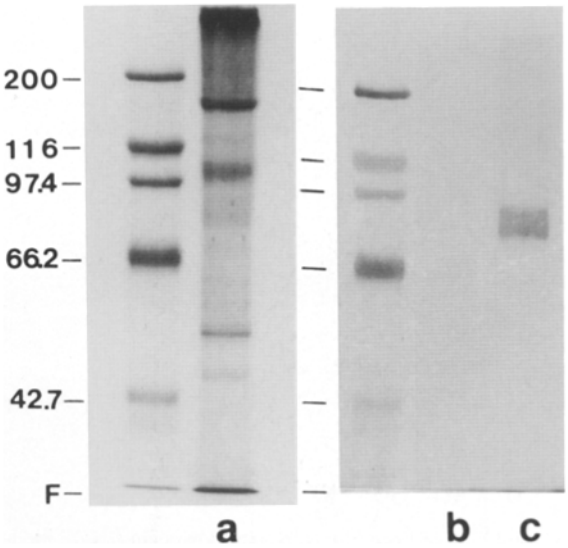

Figure 5. Immunoblots of 82-kD protein. The acetone-precipitated sample (~30–40 μg protein) was separated on 7.5% SDS-polyacrylamide gel under nonreducing conditions. Part of the gel was stained with Coomassie brilliant blue (lane *a*). The remaining gel was blotted electrophoretically onto a nitrocellulose sheet. Proteins on the sheet were immunostained with preimmune serum (lane *b*) and antiserum against NOF binding protein (lane *c*). The left lanes of each panel show the positions of marker proteins.

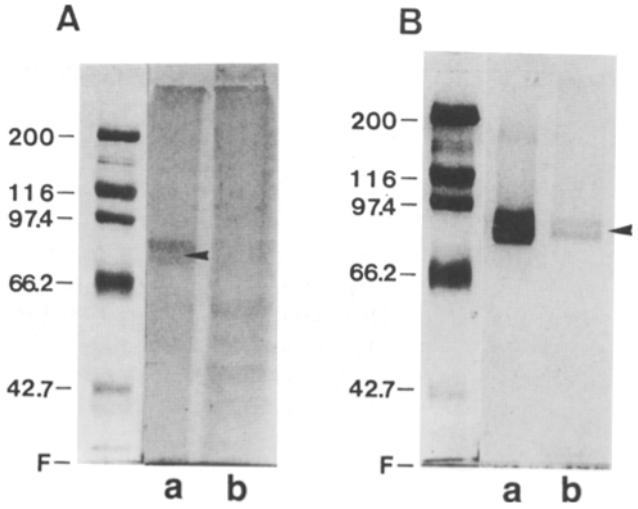

Figure 6. Detection of NOF binding protein (82 kD protein) in 10-d embryonic retina. (*A*) The P_2 fraction of 10-d embryonic retinas (40 retinas) was solubilized with 10 mM Tris-HCl (pH 7.5), 0.15% NaCl and 1% Triton X-114, and subjected to phase partitioning as described under Materials and Methods. The proteins in each phase were precipitated with 6-vol of acetone at −20°C overnight. The proteins precipitated from the detergent and aqueous phases (~50 μg protein of each phase) were separated on 7.5% SDS-gel under nonreducing conditions. The blotted sheet was incubated with NOF, washed, and then incubated with anti-NOF serum. (Lane *a*) Detergent phase; (lane *b*) aqueous phase. (*B*) An acetone-precipitated sample from a gizzard membrane extract (~60 μg protein) (lane *a*) and an acetone-precipitated sample from the detergent (Triton X-114) phase prepared from 10-d embryonic retinas (~30 μg protein) (lane *b*) were separated on 7.5% SDS-gel under nonreducing conditions, and then blotted electrophoretically onto a nitrocellulose sheet. Proteins on the sheet were immunostained with antiserum against NOF binding protein. The left lanes show the positions of marker proteins. (*Arrowheads*) Bands of 82-kD protein.

The distributions of NOF binding protein in the muscular tissues and CG from chicken embryos were also examined using antiserum against the NOF binding protein. Frozen sections of each tissue were examined by indirect immunohistochemical staining. The surface membranes of skeletal muscles from 18-d embryos and CG from 8-d embryos were strongly stained with antiserum against NOF binding protein. Intraganglionic fibrous structures were also observed in CG (data not shown).

NOF Binding Protein in Developing Chicken Retinas

We examined whether the NOF-binding protein (82-kD protein) identified in gizzard muscle is also present in neural tissues. Membrane spanning proteins are thought to be partitioned into detergent (Triton X-114) phase (1), so we prepared the membrane fraction from chicken embryonic retinas and carried out phase-partition experiments with Triton X-114. The P_2 fraction of 10-d embryonic retinas was solubilized with 1% Triton X-114, and the partition of the proteins between the detergent and aqueous phases was analyzed by ligand blotting and immunoblotting. As expected, on ligand blotting, a band of 82 kD was observed in the detergent phase

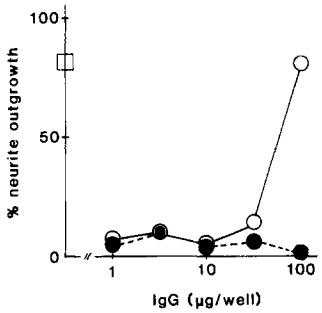

Figure 7. Neutralization of inhibition by antibody against NOF binding protein. PORN-wells precoated with NOF (100 ng protein/well) (96-multiwells) were exposed to various amounts of antibody against NOF binding protein (IgG fraction) (○) or control IgG (●) with a constant amount of purified NOF binding protein (100 ng protein/well). CG neurons were cultured in the wells and neurite extension was assayed after 5 h.

(Fig. 6 *A*, lane *a*), while the aqueous phase gave several bands other than the 82-kD band (Fig. 6, *A*, lane *b*). Antiserum against NOF binding protein also cross-reacted with a retinal 82-kD protein in the detergent phase from 10-d embryonic retinas (Fig. 6 *B*, lane *b*). The 82-kD band seemed to be a doublet band, like the NOF binding protein from gizzard muscle (Fig. 6 *B*, lane *a*). We also observed that NOF molecules in the retinas were not partitioned into the detergent (Triton X-114) phase. The inhibition assay of membrane extracts from 10-d embryonic retinas was carried out after Triton X-114 phase partition. Dose dependent inhibition was observed when increasing concentrations of Triton X-114 extract were incubated with partially purified NOF (100 ng protein/well). About 20% of the total protein was recovered in the detergent phase, but the inhibitory activity of this phase was ~10-fold that of the aqueous phase (data not shown). Approximately 80% of the activity was recovered in the detergent phase. These data suggest that the NOF-binding proteins (82-kD proteins) in chicken gizzard muscle and embryonic retina are identical or very similar proteins. Comparison of the ID_{50} values of the NP-40 extracts by the inhibition assay showed that the extracts of 6–8-d embryonic retinas had 70–80% of the activity of the gizzard muscle extract, whereas that of 18-d embryonic retina had much less activity.

Biological Activities of Antibody against NOF Binding Protein

We examined the biological activities of antibody against NOF binding protein using its IgG fraction prepared as described in Materials and Methods. A constant amount of purified NOF binding protein (100 ng protein/well) was incubated with serial dilutions of antibody against NOF binding protein (IgG fraction) or control IgG. The mixtures were introduced into PORN-wells precoated with NOF (100 ng protein/well), and neurite outgrowth from CG neurons seeded into these wells was measured after 5 h culture. The inhibition of neurite outgrowth by the exogenous NOF binding proteins was neutralized by antibody against NOF binding protein in a dose dependent manner, whereas control IgG had no effect (Fig. 7). Purified NOF binding protein was transferred to nitrocellulose sheets and incubated with NOF in the presence of serial dilutions of antibody against NOF-binding protein or control IgG. Then bands of protein were detected with monoclonal antibody to NOF. Staining of the 82-kD band was found to decrease in the presence of increasing amounts (10–100 μg protein/ml) of antibody against NOF binding protein, but not in the presence of control IgG (100 μg protein/ml) (Fig. 8). We then examined whether antibody against NOF binding protein inhibited neurite outgrowth from cultured CG or retinal neuron. Neurite outgrowth from retinal explants (Fig. 9 *A*) or CG neurons (Fig. 9 *B*) elicited by NOF was inhibited by the addition of antibody against NOF binding protein, but not of control IgG. The antibody against NOF binding protein did not have a toxic effect or cause detachment of neurons. A constant amount (30 μg protein/well) of antibody against NOF binding protein or control IgG (30 μg protein/well) was added to a series of wells that had been coated with various amounts of NOF and neurite outgrowth from CG neurons was examined. The promotion of neurite outgrowth by various amounts of NOF was found to be markedly inhibited by antibody against NOF binding protein (Fig. 10 *A*). However, no effects were observed when mouse laminin was used instead of NOF (Fig. 10 *B*). The addition of more antibody against NOF binding protein (100 μg protein/well) also blocked neurite outgrowth at a high (1 μg/well) concentration of NOF, but did not inhibit that with laminin (1 μg/well). These data suggest that antiserum against the NOF binding protein specifically blocked neurite outgrowth by neutralizing the NOF binding protein. Thus, we conclude that extension of neurites occurs as the result of recognition by NOF binding proteins on growth cones of the neurite promoting domain of NOF molecules.

Immunohistochemistry of Developing Retinas

The localization of NOF binding protein in developing retinas was studied by immunohistochemical examination of frozen sections from embryonic retinas. Strong staining with antiserum against NOF binding protein was observed in the optic nerve layers with intraretinal fibrous structures of 8-d

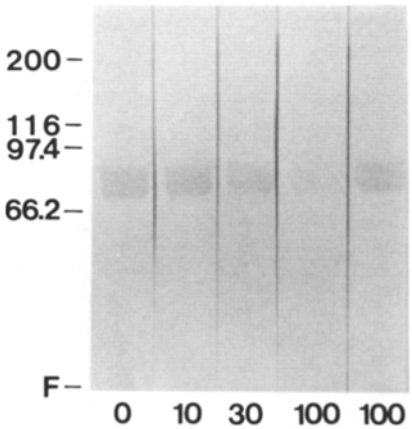

Figure 8. Inhibition of ligand blots by antibody against NOF binding protein. The purified NOF binding protein (~10 μg protein) was separated on 7.5% SDS-gel and transferred to a nitrocellulose sheet. The sheet was incubated with NOF (ligand NOF) and various amounts of antibody against NOF binding protein (IgG fraction) (first four lanes, *from left*) or control IgG (last lane *from left*) and then bands were detected with NOF monoclonal antibody (1-4D). Figures under lanes show protein concentrations of IgG in micrograms.

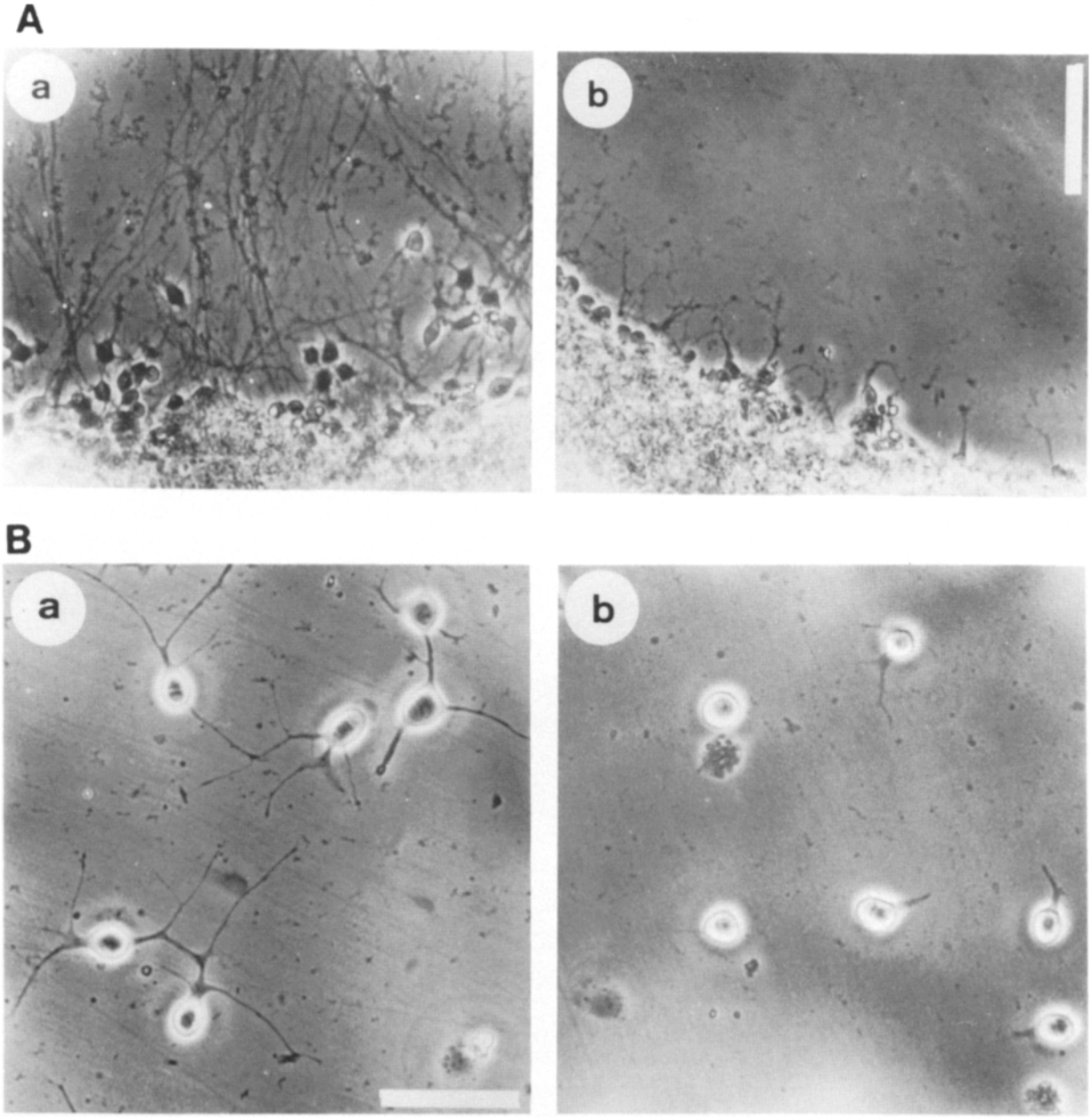

Figure 9. Inhibition by antibody against NOF binding protein of neurite outgrowth from cultured retina or CG neurons elicited by NOF. (*A*) Retinal explants from 8-d embryos were placed in PORN-wells precoated with NOF (1,000 ng protein/well) (24-well plates). The wells were incubated at 37°C for 24 h in culture medium containing (*a*) control IgG or (*b*) antibody against NOF binding protein (IgG fraction, 300 μg protein/well). The cultured retinas were then photographed with a phase-contrast microscope. (*B*) Dissociated CG neurons from 8-d-old embryos were seeded in PORN-wells precoated with NOF (100 ng protein/well) (96-well plates). The wells were incubated at 37°C for 5 h in culture medium containing (*a*) control IgG or (*b*) antibody against NOF binding protein (30 μg protein/well). The cultured CG neurons were then photographed with a phase-contrast microscope. Bars, 150 μm.

embryonic retina (Fig. 11 *b*), but no reaction with the antibody was observed in 18-d embryonic retina (Fig. 11 *d*).

To confirm that NOF binding protein in the retina decreases during development, we measured the amounts of NOF-binding protein in the detergent (Triton X-114) phase of extracts of embryonic retinas of various ages by ligand blotting and inhibition assay. A band of 82 kD was clearly detected in extracts of 8–11-d retinas, but at later stages its intensity decreased with age (Fig. 12 *A*). The inhibitory activity in the detergent phase was also maximal in preparations from 8- and 11-d embryonic retinas and was progressively less in those from 14- and 18-d embryos (Fig. 12 *B*). Immunoblot analysis using antiserum against NOF binding protein in the detergent phases from retinas of various ages gave similar results to those obtained by ligand blotting (data not shown).

Discussion

In this work, we purified an NOF binding protein from

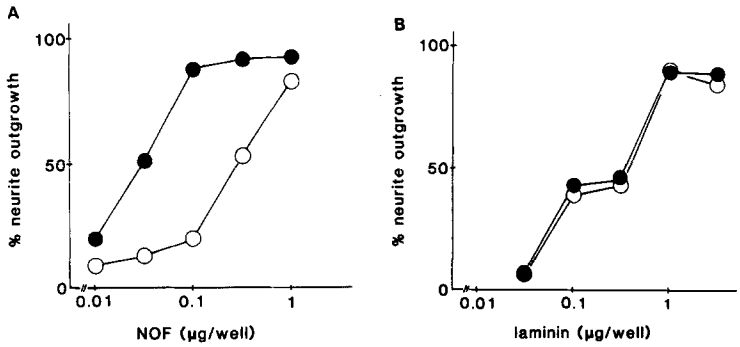

Figure 10. Inhibitory effect of antibody against NOF binding protein on the neuritic activities of NOF and mouse laminin. CG neurons from 8-d chick embryos were cultured and the neurite extension was measured after 5 h. The percentage of total surviving neurons bearing neurites was determined. (*A*) A constant amount (30 μg protein/well) of antibody against NOF binding protein (○) or control IgG (●) was added to a series of wells that had been coated with various amounts of NOF. (*B*) A constant amount (30 μg protein/well) of antibody against NOF binding protein (○) or control IgG (●) was added to wells coated with various concentrations of mouse laminin.

chicken gizzard muscle to apparent homogeneity on SDS-PAGE and prepared a specific antibody against it to study the properties of the NOF binding protein and its physiological roles. The purity achieved was ~300-fold that of the crude NP extract, as judged by inhibition assay. However, this estimate of purification is inaccurate because the crude NP-40 extract contained some NOFs that formed a complex(es) with the NOF binding protein and so caused decrease in the apparent amount of free NOF binding protein.

We also found that a similar 82-kD protein is present in developing chicken retina. Antiserum against the 82-kD protein from gizzard muscle cross-reacted with the retinal 82-kD protein, which appeared to be a doublet like that in gizzard muscle. These findings suggest that the NOF binding proteins in the chicken gizzard muscle and chick embryonic retina are identical or very similar proteins. The NOF binding protein was estimated to constitute ~0.1–0.05% of the protein in the retinal membrane fraction. The activity of the NOF binding protein in the crude extract from embryonic retina could not be compared with that in the extract of giz-

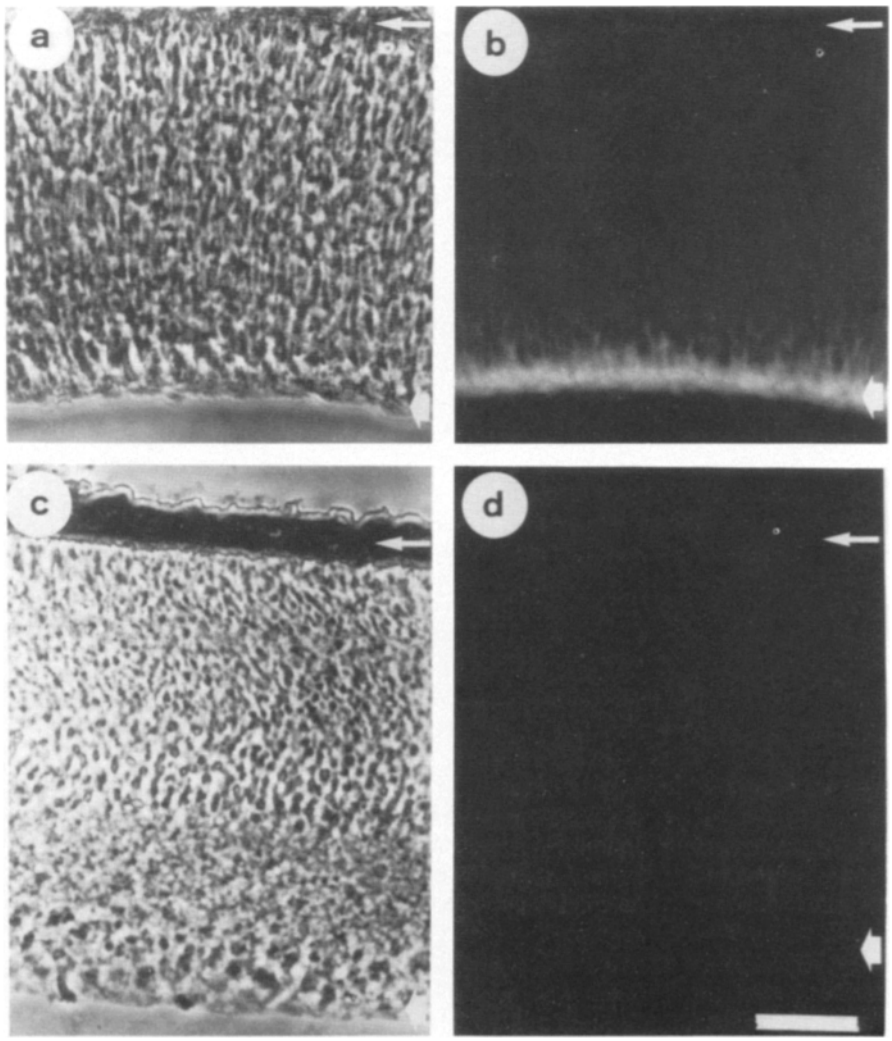

Figure 11. Immunofluorescent staining of chicken embryonic retinas. Thin sections (15 μm) of embryonic retinas were fixed and stained with antiserum against NOF binding protein. 8-day (*a* and *b*) and 18-d (*c* and *d*) embryonic retinas were photographed with a phase-contrast microscope (*a* and *c*), and the same fields were stained by the indirect immunofluorescent procedure (*b* and *d*). The bar represents 200 μm. (*Long arrow*) Pigment epithelium layer; (*short arrow*) optic fiber layer.

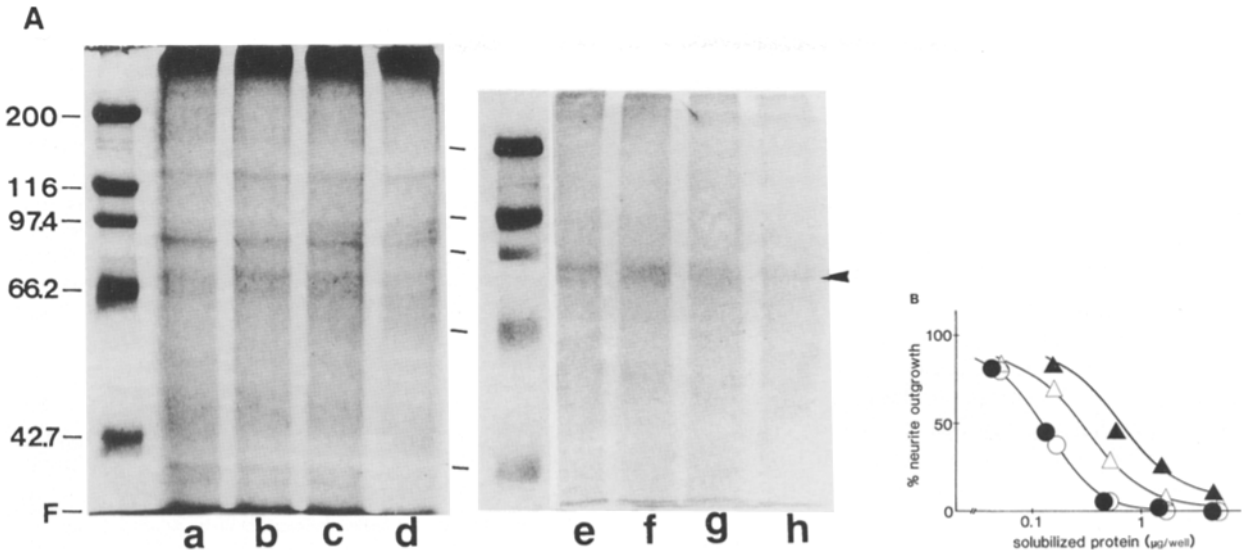

Figure 12. Changes in NOF binding protein during retinal development. (*A*) The membrane fractions from 8- (lanes *a* and *e*), 11- (lanes *b* and *f*), 14- (lanes *c* and *g*), and 18- (lanes *d* and *h*) day embryonic retinas were partitioned into the Triton X-114 phase and the proteins in this phase were precipitated with sixfold excess of acetone. The precipitated proteins (~30 μg) were separated on 7.5% SDS-gel under nonreducing conditions. Part of the gel (lanes *a*, *b*, *c*, and *d*) was stained with Coomassie brilliant blue. The rest of the gel (lanes *e*, *f*, *g*, and *h*) was blotted electrophoretically onto a nitrocellulose sheet. The NOF binding proteins were detected by ligand blotting. The left lane of each panel shows the positions of marker proteins. (*Arrowhead*). Bands of NOF binding protein (*B*) The proteins precipitated from 8- (●), 11- (○), 14- (△), and 18- (▲) day embryonic retinas in *A* were solubilized with 10 mM Tris acetate (pH 8.0), 1 mM EDTA and 0.5% NP-40. PORN-wells precoated with NOF (100 ng protein/well) were exposed to various amounts of solubilized proteins in the detergent phases. Inhibition assay using CG neurons was carried out. Points are means for samples obtained in three independent experiments. Variations from the means were <10%.

zard muscle by inhibition assay because some of the NOF binding protein in the gizzard muscle was present as a complex with NOF, whereas the NOF binding protein in the extract of embryonic retina was almost free of NOF as judged by immunoblotting. Comparison of the staining intensities in ligand blots or immunoblots of the NP-40 extracts indicated that extracts of 6–8-d embryonic retinas contained <10% of the NOF binding protein of gizzard muscle.

Kleinmann et al. (12) reported that NG108-15 hybrid cells extend neurites in the presence of laminin and that ^{125}I-labeled laminin binds to three membrane proteins of 67, 110, and 180 kD. The 67-kD protein is responsible for cell attachment (5), and the 110- and 180-kD proteins are both involved in neurite outgrowth induced by laminin. We have reported that the 82-kD protein (NOF binding protein) does not affect cell attachment (19). Integrins, receptors for several extracellular matrix proteins, have been reported to participate in neurite outgrowth from some neurons (2, 20). The NOF binding protein differs from integrins in the following characters: (*a*) as molecular size is different from those of the alpha and beta subunits of integrins, which are in the range of 120–200 kD; (*b*) monoclonal antibody against the beta-1 subunit of integrins does not cross-react with the NOF binding protein, and polyclonal antibody against the NOF binding protein does not react with the retinal or gizzard beta-1 integrin subunit (data not shown); (*c*) the NOF binding protein did not bind to the alpha-subunit of integrins, but the beta-1 integrin subunit bound to the alpha-subunit of integrins (data not shown); (*d*) the binding of integrins to laminin requires calcium ion (2, 20), whereas the binding of the NOF binding protein to NOF does not require calcium ion or magnesium ion. (*e*) Laminin does not bind to NOF binding protein (19) and antiserum against NOF binding protein did not inhibit the neuritic activity of laminin. These data suggest that the NOF binding protein is a novel membrane protein that recognizes the neurite promoting domain of NOF.

A monoclonal antibody against the chick integrin beta-1 subunit slightly inhibited neurite outgrowth induced by NOF. However, when a crude NP-40 extract from gizzard muscle membranes was applied to an NOF-affinity column and the column was developed with 1 M NaCl, three bands of 160, 110, and 82 kD were detected by SDS-PAGE. Material in the 110-kD band reacted only with anti-beta-1 monoclonal antibody, and that in the 82-kD band reacted only with antiserum against the 82-kD protein (data not shown). These data indicate that integrins somehow influence neurite outgrowth induced by NOF, but that an 82-kD membrane protein mainly reacts with NOF.

The antibody against NOF binding protein was used to examine the distribution and developmental change of NOF binding protein in chick retina. In indirect immunofluorescence studies, optic nerve layers with intraretinal fibrous structures in early embryonic retinas were stained by antibody against the NOF binding protein. However, this staining was not seen in late embryonic retinas (18-d-old). Moreover, measurement of the amount of NOF binding protein in embryonic retinas of various ages by ligand blotting and inhibition assay showed that the amount of NOF binding protein was maximal in retinas of 8–11-d embryos and then decreased with age. We have reported that neurite outgrowth induced by NOF is maximal in cultured retinas from 8-d-old embryos, and that later the neurons gradually lose responsiveness to NOF (11). Thus, during development, decrease in the NOF binding protein molecules is correlated with loss

of responsiveness of the retinas to NOF. In immunohistochemical studies on 8-d embryonal retinas, anti-NOF serum stained the inner limiting membrane and the neuroepithelial cells of the retina, and the optic fissure and tract. This distribution of NOF is roughly similar to that of laminin (3). NOF binding protein is also expressed throughout the optic pathway within the retina and optic nerve during early development. In cultures of retina, the neurites induced by NOF were stained with anti-82-kD protein serum (data not shown), suggesting that NOF binding protein is present on the growing axon. Cohen et al. (3, 4) reported that neurite extension from retinal ganglion cells induced by laminin occurs in a discrete developmental period and that loss of the response is parallel with decrease in the receptor numbers on both the cell bodies and neurites of ganglion cells. The relation of the NOF binding protein with their laminin receptors is unknown because they did not report the molecular size of the laminin receptor.

We do not know the physiological role(s) of the NOF binding protein in muscular tissues. Interaction of myoblasts with extracellular matrix substances is reported to be essential for myogenic differentiation (17). Since the muscles of late embryos express NOF binding protein diffusely, NOF binding protein may play a role in myogenic differentiation. s-Laminin, a laminin-like protein, is located in the synaptic cleft of the neuromuscular junction (10) and has been implicated in the formation or stabilization of synapses. NOF and NOF binding protein in muscles may also function in formation or stabilization of synapses. Recently, we observed that NOF binding protein is expressed in a specific area of the brain in early embryos. Cloning of NOF binding protein cDNA is in progress to examine whether the neural NOF-binding protein is identical with that of muscles. Further experiments are needed to elucidate the structure and functions of the NOF binding protein during retinal or brain development and also to confirm that the 82-kD protein is an NOF receptor.

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References

1. Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256:1604–1607.
2. Bozyczko, D., and A. F. Horwitz. 1986. The participation of a putative cell surface receptor for laminin and fibronectin in peripheral neurite extension. *J. Neurosci.* 6:1241–1251.
3. Cohen, J., J. F. Burne, J. Winter, and P. Barnett. 1986. Retinal ganglion cells lose response to laminin with maturation. *Nature (Lond.).* 332: 465–467.
4. Cohen, J., V. Nurcombe, P. Jeffrey, and D. Edgar. 1989. Developmental loss of functional laminin receptors on retinal ganglion cells is regulated by their target tissues, the optic tectum. *Development (Camb.).* 107: 381–387.
5. Graf, J., Y. Iwamoto, M. Sasaki, G. R. Martin, H. K. Kleinmann, F. A. Robey, and Y. Yamada. 1987. Identification of an amino acid sequence in laminin mediating cell attachment, chemotaxis, and receptor binding. *Cell.* 48:989–996.
6. Hayashi, Y., and N. Miki. 1985. Purification and characterization of a neurite outgrowth factor from chicken gizzard smooth muscle. *J. Biol. Chem.* 260:14269–14278.
7. Hayashi, Y., H. Higashida, C.-H. Kuo, and N. Miki. 1984. Antiserum against neurite outgrowth factor in chick gizzard extract and its inhibitory effect on neuritic response in cultured ciliary neurons. *J. Neurochem.* 42:504–512.
8. Hayashi, Y., H. Taniura, and N. Miki. 1987. Interaction of monoclonal antibodies with a neurite outgrowth factor from chicken gizzard extract. *Dev. Brain Res.* 35:11–19.
9. Hopkins, J. M., T. S. Ford-Holevinski, J. P. McCoy, and B. W. Agranoff. 1985. Laminin and optic nerve regeneration in the goldfish. *J. Neurosci.* 5:3030–3038.
10. Hunter, D. D., S. Vandana, J. P. Merlie, and J. R. Sanes. 1989. A laminin-like adhesive protein concentrated in the synaptic cleft of the neuromuscular junction. *Nature (Lond.).* 338:229–234.
11. Kato, S., K. Negishi, Y. Hayashi, and N. Miki. 1983. Enhancement of neurite outgrowth and aspartate-glutamate uptake systems in retinal explants cultured with chick gizzard extract. *J. Neurochem.* 40:929–938.
12. Kleinmann, H. K., R. C. Ogle, F. B. Cannon, C. D. Little, T. H. Sweeney, and L. Luckenbill-Edds. 1988. Laminin receptors for neurite formation. *Proc. Natl. Acad. Sci. USA.* 85:1282–1286.
13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).*227:680–685.
14. Letourneau, P. C., A. M. Madsen, S. L. Palm, and L. T. Furcht. 1988. Immunoreactivity for laminin in the developing ventral longitudinal pathway of the brain. *Dev. Biol.* 125:135–144.
15. Liesi, P. 1985. Do neurons in the vertebrate CNS migrate on laminin? *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1163–1170.
16. Liesi, P. 1985. Laminin immunoreactive glia distinguish regenerative adult CNS systems from non-regenerative ones. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2505–2511.
17. Menko, A. S., and D. Boettiger. 1987. Occupation of the extracellular matrix receptor, integrin, is a control point for myogenic differentiation. *Cell.* 51:51–57.
18. Miki, N., Y. Hayashi, and H. Higashida. 1981. Characterization of chick gizzard extract that promotes neurite outgrowth in cultured ciliary neurons. *J. Neurochem.* 37:627–633.
19. Taniura, H., Y. Hayashi, and N. Miki. 1988. An 82-kilodalton membrane protein that inhibits the activity of neurite outgrowth factor. *J. Neurochem.* 50:1572–1578.
20. Tomaselli, K. J., L. F. Reichardt, and J. L. Bixby. 1986. Distinct molecular interactions mediate neuronal process outgrowth on nonneuronal cell surfaces and extracellular matrices. *J. Cell Biol.* 103:2659–2672.
21. Ruoslahti, E., and M. D. Pierschbacher. 1986. Arg-Gly-Asp: a versatile cell recognition signal. *Cell.* 44:517–518.
22. Ruoslahti, E. 1988. Fibronectin and its receptors. *Annu. Rev. Biochem.* 57:375–413.