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Association of Nerve Growth Factor Receptors with the Triton X-100 Cytoskeleton of PC12 Cells[1]

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Abstract

Triton X-100 solubilizes membranes of PC12 cells and leaves behind a nucleus and an array of cytoskeletal filaments. Nerve growth factor (NGF) receptors (10% of those found in intact cells) are associated with this Triton X-100-insoluble residue. Two classes of NGF receptors are found on PC12 cells which display rapid and slow dissociating kinetics. Although rapidly dissociating binding is predominant (>75%) in intact cells, the majority of binding to the Triton X-100 cytoskeleton is slowly dissociating (>75%). Rapidly dissociating NGF binding on intact cells can be converted to a slowly dissociating form by the plant lectin wheat germ agglutinin (WGA). This lectin also increases the number of receptors which associate with the Triton X-100 cytoskeleton by more than 10-fold. ^{125}I-NGF bound to receptors can be visualized by light microscopy autoradiography in Triton X-100-insoluble residues of cell bodies, as well as growth cones and neurites. The WGA-induced association with the cytoskeleton, however, is not specific for the NGF receptor, since >90% of cell surface glycoprotein receptors for WGA become associated with Triton X-100-insoluble material at lectin concentrations greater than 33 μg/ml. Concentrations of WGA which change the Triton X-100 solubility of membrane glycoproteins are similar to those required to alter the kinetic state of the NGF receptor. Both events may be related to the crossbridging of cell surface proteins induced by this multivalent lectin.

Plasma membrane proteins were once thought to diffuse freely in the plane of the lipid bilayer. However, a good deal of evidence has accumulated in recent years indicating that many membrane proteins associate with a subcortical cytoskeletal network (Branton et al., 1981; Mescher et al., 1981; Pober et al., 1981). This transmembrane interaction has many important consequences for cellular function. Connections between the cytoskeleton and the membrane are important for controlling cell shape and locomotion (Hellewell and Taylor, 1979; Stossel, 1982) and may regulate many other dynamic membrane processes such as patching, capping, and internalization of cell surface antigens (Bourguignon and Singer, 1977; Condeelis, 1979). Furthermore, cytoskeletal regulation of membrane protein mobility (Schlessinger et al., 1976; Koppel et al., 1981; Carraway et al., 1983) allows for the formation of specialized membrane domains, as demonstrated by the clustering of acetylcholine receptors at synaptic junctions (Prives et al., 1982). In the erythrocyte, proteins responsible for linking membrane proteins to actin microfilaments have been identified and purified (Bennett, 1982); however, in non-erythroid cells, the biochemistry of membrane-cytoskeletal interactions is still poorly understood. In the last few years, though, proteins similar in nature to erythroid spectrin and ankyrin have been identified in other cell types (Burridge et al., 1982b), and novel proteins which may play a role in transmembrane linkage have also been found (Burridge et al., 1982a; Carraway et al., 1983).

Nerve growth factor (NGF), a hormone which plays an essential role in neuronal development (Thoenen and Barde, 1980; Yankner and Shooter, 1982), interacts with specific cell surface receptors on a cultured pheochromocytoma cell line, PC12 (Herrup and Thoenen, 1979; Landreth and Shooter, 1980). Receptor binding initiates a sequence of events which transforms PC12 cells into a cell phenotype resembling a sympathetic neuron (Greene and Tischler, 1976). Two types of NGF receptors with high and low affinity have been identified on responsive cells (Sutter et al., 1979a; Landreth and Shooter, 1980). The two receptors can be easily distinguished by their dissociation kinetics, as ^{125}I-NGF is released 10 to 100 times more slowly from high affinity than from low affinity receptors. Previously, Schechter and Bothwell (1981) found that ^{125}I-NGF, bound to slowly dissociating receptors, was resistant to Triton X-100 solubilization whereas rapidly dissociating binding was completely solubilized. They proposed that an association of slowly dissociating receptors with the cytoskeleton was responsible for their Triton X-100 insolubility.

Vale and Shooter (1982, 1983) later showed that rapidly dissociating NGF-receptor complexes could be converted to a slowly dissociating form by the lectin wheat germ agglutinin (WGA) (Vale and Shooter, 1982) or by anti-NGF antibodies (Vale and Shooter, 1983). The change in the kinetic state of the receptor was accompanied by a conversion of the NGF-receptor complex to a Triton X-100-insoluble form. These results suggest that an association of the NGF receptor with the cytoskeleton is correlated with a particular binding state. Recent studies by Jesaitis et al. (1984) have also shown that the conversion of the N-formyl chemotactic peptide receptor to a slowly dissociating form on granulocytes occurs concomitantly with a transient association with the cytoskeleton. In the current study, we demonstrate that the Triton X-100-insoluble residue of PC12 cells contains NGF receptors capable of binding NGF. The slowly dissociating kinetic form of the receptor is primarily found in association with the Triton X-100 cytoskeleton, and the extent of ^{125}I-NGF binding to the cytoskeletons can be greatly enhanced by prior treatment of the cells with WGA.

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Materials and Methods

Materials

WGA and concanavalin A (Con A) were purchased from Vector Laboratories. Triton X-100, lactoperoxidase, and DNase I (DN CL) were obtained from Sigma Chemical Co. Glucose oxidase was purchased from Calbiochem-Behring. NGF (the β subunit of the 7 S NGF complex) was purified as previously described (Mobley et al., 1976).

Methods

Iodinations. NGF was radiolabeled with ^{125}I by a lactoperoxidase procedure to a specific activity of 50 to 90 cpm/pg as described by Sutter et al. (1979a). ^{125}I-NGF was used within 3 weeks of its iodination. ^{125}I-WGA (25 to 30 cpm/pg) was prepared by the Sutter et al. (1979a) protocol except that 250 μg, instead of 50 μg, of protein was used in the reaction mixture. Iodination of PC12 cell surface proteins was performed according to the protocol of Hynes (1973) using a lactoperoxidase/glucose oxidase coupled reaction. Labeled cells were separated from free ^{125}I by four successive 10-sec centrifugations at 10,000 × *g* in a microfuge with washes of Dulbecco's phosphate-buffered saline (PBS) containing 10 mM KI and 1 mM phenylmethylsulfonyl fluoride (PMSF).

Cell culture and harvesting. PC12 cells were grown in 100-mm tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 5% horse serum (DMEM + S) in an atmosphere of 88% air and 12% CO_2. For binding assays, dishes were washed twice with 10 ml of PBS containing 1 mg/ml each of glucose and bovine serum albuminin (BSA) (PBS/BSA/glucose) and were harvested by trituration in 10 ml of the same buffer. The concentration of cells was estimated using a hemocytometer and was adjusted to between 1 and 2 × 10^6 cells/ml. Experimentation began immediately after removal of cells from dishes.

Preparation of Triton X-100 cytoskeletons. PC12 cells (2 × 10^6/ml unless indicated) were centrifuged (500 × *g*; 5 min) and resuspended with a 5- or 10-ml plastic pipette in one-half of their original volume in 0.5% Triton X-100 in 20 mM Tris-HCl, pH 7.4, 4 mM $MgCl_2$, 300 mM sucrose, and 1 mM PMSF (Triton X-100 extraction buffer) for 5 min at 4°C. This is the same buffer used by Ben Ze'ev et al. (1979) for extracting fibroblasts. Since cytoskeletal proteins are prominent components that remain after detergent extraction, we use the term "Triton X-100 cytoskeleton" in reference to cellular material that remains insoluble after adding detergent and pellets at low centrifugal speeds. Triton X-100 cytoskeletons were washed twice in PBS/BSA by centrifugations at 1000 × *g* for 5 min at 4°C to remove the majority of the Triton X-100 detergent. The cytoskeleton pellet was then resuspended in its original volume with PBS/BSA for a final concentration of 2 × 10^6 Triton X-100 cytoskeletons/ml. In the case of Triton X-100 cytoskeletons obtained from WGA-treated cells, WGA (50 μg/ml) was included in the buffer used to resuspend the cytoskeleton pellet.

Enucleation of PC12 cells. PC12 cells were subcultured for 1 to 2 days in 25-cm^2 flasks on extracellular matrix obtained from confluent bovine corneal endothelial cells. Enucleation was performed by first incubating cells with 8 μg/ml of cytochalasin in DMEM plus 10% calf serum with sufficient medium to fill the flask. The flasks were placed in a JA-10 rotor containing a 200-ml water cushion (37°C) and were centrifuged at 7,000 to 11,000 × *g* for 15 min in a Beckman model J-21C centrifuge. The medium containing sheared nucleoplasts was aspirated, and cytoplasts were incubated with 5 ml of Ca^{2+}, Mg^{2+}-free PBS for 20 min at 37°C. Cytoplasts were removed by trituration and were centrifuged (500 × *g*; 5 min) and resuspended in binding buffer. This procedure results in >85% enucleation. In parallel with the preparation of cytoplasts, a control preparation of intact cells underwent the same treatment with the exception of cytochalasin B treatment.

^{125}I-NGF binding. ^{125}I-NGF was added in PBS/BSA/glucose or PBS/BSA to cells and Triton X-100 cytoskeletons, respectively. All experiments were conducted in polyethylene tubes. After a given incubation, triplicate aliquots (100 μl) were layered over 200 μl of 0.15 M sucrose in binding buffer in 400-μl microfuge tubes and centrifuged for 30 sec at 10,000 × *g* in a Microfuge. Bound and free ^{125}I-NGF were separated by freezing the Microfuge tube in a dry ice/ethanol bath and cutting it just above the cell pellet. Nonspecific ^{125}I-NGF binding was determined by incubating cells or cytoskeletons with ^{125}I-NGF and 10 μg/ml of unlabeled NGF. Nonspecific binding was subtracted from the total bound radioactivity, and specific binding is reported for all experiments. Nonspecific binding to whole cells was generally less than 10% of the total. Triton X-100 cytoskeletons from WGA-treated and untreated cells displayed nonspecific bindings of 15% and 50%, respectively. Slowly dissociating binding was determined by removing 0.4 ml of the suspension of cells or cytoskeletons containing ^{125}I-NGF to a tube containing 10 μl of a 0.5-mg/ml solution of unlabeled NGF (final concentration of 8 μg/ml of unlabeled ligand) and incubating the sample on ice for 30 min. The amount of ^{125}I-NGF that remains associated with the cells or cytoskeletons after dissociation was determined by the same centrifugation assay already described and was corrected for nonspecific binding. For intact cells, Triton X-100-insoluble binding was determined by centrifuging triplicate 100-μl aliquots through 200 μl of the previously described Triton X-100 extraction buffer for 30 sec at 10,000 × *g* in a Microfuge. Radioactivity which pelleted was considered Triton X-100 insoluble and had Triton X-100-insoluble nonspecific binding subtracted.

Light microscopic autoradiography. Cells were grown for 8 days in 50 ng/ml of NGF in DMEM + S on poly-L-lysine (1 mg/ml, type IV, Sigma)-coated polystyrene coverslips (Lux) in multiwell culture dishes (Lux). Cells were plated at a density of 2.5 × 10^3 cells/cm^2 to minimize crowding of processes and cell bodies. On day 9 cells were washed three times for 1 hr with NGF-free DMEM + S to remove surface bound NGF. ^{125}I-NGF (1 nM) in PBS/BSA/glucose was added in the presence or absence of WGA (50 μg/ml) for 30 min at 37°C. Medium was then removed and 0.5% Triton X-100 extraction buffer with 1 mM PMSF was added at 4°C for 5 min, and then the cells were washed three times for 1 min in Triton X-100-free extraction buffer. The extracted cells were fixed overnight in 1% paraformaldehyde, 2% glutaraldehyde and 5% sucrose in PBS. The following day the coverslips were washed three times in PBS/glucose and twice in distilled H_2O just prior to dipping in emulsion.

Autoradiographic procedures were essentially those described by Kopriwa and Leblond (1962). Coverslips were dipped into Kodak NTB2 nuclear emulsion, diluted 1:1 with distilled H_2O, and allowed to dry before being left to expose at 4°C for 17 days in the dark. The exposed coverslips were developed in Kodak D-19 developer for 2 min at 21°C, washed for 15 sec in distilled H_2O, fixed for 2 min in Kodak Rapid Fixer, and washed with filtered water for 15 min. The air-dried coverslips were mounted on glass slides with 50% glycerine and viewed with a Zeiss Universal microscope under phase and brightfield optics at × 200 to 400 magnification.

Scanning electron microscopy. Cells were grown and treated with NGF as before for light microscopy. On day 9, cells were extracted in 0.5% Triton X-100 extraction buffer for 5 min at 4°C, washed three times in Triton-X-100 minus extraction buffer, and fixed with 1% paraformaldehyde, 2% glutaraldehyde and 5% sucrose in PBS. After 5 min fixation, cells were washed three times for 1 min in PBS/0.1% glucose, post-fixed in 2% osmium in 0.1 M sodium phosphate (pH 7.4) for 30 min, rinsed three times more in PBS/glucose, and then dehydrated through an acetone series. Coverslips with their overlay of extracted, fixed cells were critical point dried according to the method of Debault (1973) and then was coated with a 100-Å-thick layer of gold in a sputtering device and viewed in an ISI-40 scanning electron microscope.

Results

Binding of ^{125}I-NGF to PC12 Triton X-100 cytoskeletons. Schechter and Bothwell (1981) have shown that a portion of cell-bound ^{125}I-NGF is not solubilized by Triton X-100. We have repeated this experiment and similarly found that about 15 to 20% of ^{125}I-NGF binding pelleted with Triton X-100-insoluble material (Table I). We then investigated whether Triton X-100 cytoskeletons which were washed free of detergent and resuspended in PBS/BSA contain functional NGF receptors. NGF receptors in this Triton X-100-insoluble fraction would have to be attached to a very large structure in order for them to pellet at low centrifugal speeds (500 to 1000 × *g* for 5 min). Table I shows that Triton X-100 cytoskeletons prepared in this manner demonstrate 5 to 10% of the NGF binding activity of the cells from which they were derived. This value is 3- to 4-fold lower than if ^{125}I-NGF were first bound to cells prior to Triton X-100 extraction.

Pretreatment of PC12 cells with WGA increases the NGF binding activity associated with the Triton X-100 cytoskeleton. As previously shown (Vale and Shooter, 1982; Grob and Bothwell, 1983) this lectin decreases NGF binding by 50% when added prior to ^{125}I-NGF, and the majority of this binding is not solubilized by Triton X-100. Table I also shows that Triton X-100 cytoskeletons prepared from WGA-treated cells contained almost 10-fold the amount of NGF binding activity as those derived from untreated cells. In fact, WGA-treated intact cells and Triton X-100 cytoskeletons derived from these cells displayed similar levels of NGF binding. Therefore, unlike WGA-

untreated cells, association of the receptor with the Triton X-100 cytoskeleton was not influenced by prior NGF binding in the presence of WGA. Receptor affinities were approximately the same in cells and Triton X-100 cytoskeletons (data not shown). ^{125}I-NGF also bound to Triton X-100 cytoskeletons obtained from chick embryonic dorsal root ganglion cells and A875 cells, and binding, once again, was dramatically increased by pretreating these cells with WGA (data not shown).

In order to cause maximal association of the NGF receptor with Triton X-100-insoluble material, WGA must be added to cells prior to detergent extraction. Table II shows that WGA addition to Triton X-100-insoluble pellets did not cause a significant increase in NGF binding activity, indicating that the lectin is not merely activating masked receptors. WGA caused some NGF binding activity to pellet from detergent-soluble supernatants, but such binding was an order of magnitude lower than that found on cytoskeletons derived from WGA-treated cells. This effect could be due to the precipitation of soluble NGF receptors or small receptor-cytoskeleton complexes by WGA, a multivalent lectin. Interestingly, WGA addition to a combination of Triton X-100-soluble and -insoluble material caused a greater increase in pelletable binding than would be predicted on the basis of the lectin's effects on Triton X-100-soluble and -insoluble fractions separately. This finding implies that WGA causes an association of soluble NGF receptors with the Triton X-100-insoluble cytoskeleton, possibly due to crosslinking.

NGF receptors associated with the Triton X-100 cytoskeleton display slow dissociation kinetics. Two species of NGF receptors are found on PC12 cells: one a high affinity, slowly dissociating subtype and the other a low affinity, rapidly dissociating subtype (Landreth and Shooter, 1980). The two species can readily be distinguished, since ^{125}I-NGF bound to rapidly dissociating receptors is completely released within 15 min upon adding excess unlabeled NGF, whereas ligand remains stably bound to the slowly dissociating receptor in this same time period. Table I shows that with 500 pM ^{125}I-NGF, approximately 25% of the total binding to whole cells was slowly dissociating. In contrast, under similar conditions, 70 to 100% of the total binding to Triton X-100 cytoskeletons was slowly dissociating. WGA increases the amount of slowly dissociating binding on cells by converting rapidly dissociating receptors into a slowly dissociating form which may or may not be exactly the same as the naturally occurring slow receptor (Vale and Shooter, 1982), and it also increases in proportion the binding of ^{125}I-NGF to the Triton X-100 cytoskeleton. Once again, this binding was primarily slowly dissociating. Thus, the association of NGF receptors with the Triton X-100 cytoskeleton is correlated with a high affinity, slowly dissociating binding state of the receptor for NGF.

The time course of the appearance of rapidly and slowly dissociating binding in Triton X-100 cytoskeletons is shown in Figure 1. With WGA-pretreated preparations, maximum levels of rapidly dissociating binding were achieved after 5 min of ^{125}I-NGF incubation. In contrast, slowly dissociating binding developed more gradually, reaching a steady state after 30 to 60 min. For Triton X-100 cytoske-

TABLE I

Binding of ^{125}I-NGF to cells and Triton X-100 cytoskeletons

PC12 cells (0.75 × 10^6/ml) were incubated with and without WGA (50 μg/ml) for 30 min at 37°C. These cell samples were divided into equal aliquots and centrifuged at 500 × *g* for 5 min. Cell pellets were then either resuspended in binding buffer or extracted with Triton X-100 extraction buffer for 5 min at 4°C. Cells or cytoskeletons were then centrifuged (1000 × *g*; 5 min at 4°C), and supernatants were removed. Pellets were washed twice and resuspended in their original volume of binding buffer. ^{125}I-NGF (580 pM) was added to cells or cytoskeletons for 45 min at 37°C, and total, slowly dissociating, and Triton X-100-insoluble (in the case of intact cells) binding were measured.

Sample	Centrifugate	^{125}I-NGF Binding				
		Total (fmol/10^6cells)	Slowly Dissociating		Triton X-100 Insoluble	
			fmol/10^6 cells	% Total	fmol/10^6 cells	% Total
−WGA	Cells	217 ± 5	57 ± 1	26	43 ± 8	20
	Cytoskeletons	12 ± 2	9 ± 3	75		
+WGA	Cells	136 ± 2	103 ± 1	76	108 ± 6	79
	Cytoskeletons	112 ± 20	80 ± 16	71		

TABLE II

Maximal association of the NGF receptor with the Triton X-100 cytoskeleton requires WGA addition to cells prior to extraction

PC12 cells (4 × 10^6/ml) were centrifuged and resuspended in Triton X-100 extraction buffer (in an equal volume) for 5 min at 4°C. Triton X-100 cytoskeletons were centrifuged at 1000 × *g* for 5 min at 4°C and the supernatant was removed and saved (Supernatant). The pellet was resuspended in a volume equal to that of the supernatant of Triton X-100 extraction buffer (Pellet). Another sample (Pellet and Supernatant) underwent identical Triton X-100 extraction; however, the pellet and supernatant were resuspended together instead of being separated after centrifugation. Triton X-100 Pellets, Supernatants and Pellets, plus Supernatants were divided equally and one-half received WGA (50 μg/ml) for 30 min at 23°C. All samples were then centrifuged (1000 × *g* for 5 min at 4°C), and the pellets were washed twice and resuspended in the original volume of PBS/BSA. An equal volume of ^{125}I-NGF in PBS/BSA was added (final concentration, 600 pM ^{125}I-NGF with 2 × 10^6/Triton X-100 cytoskeleton/ml) and incubated for 45 min at 37°C prior to assaying pelletable ^{125}I-NGF binding in a Microfuge. Nonspecific ^{125}I-NGF binding was treated in the manner described above. ^{125}I-NGF binding is expressed in femtomoles bound to material from Pellets, Supernatants, or Pellets and Supernatants from 2 × 10^6/ml of Triton X-100-extracted cells. For comparison, it is also expressed as the percentage of NGF binding to 2 × 10^6/ml of Triton X-100 cytoskeletons derived from cells treated with 50 μg/ml of WGA for 30 min at 23°C prior to detergent extraction.

Sample	^{125}I-NGF Binding			
	−WGA		+WGA	
	fmol (from 2 × 10^6 cells)	% of WGA-pretreated Cells	fmol (from 2 × 10^6 cells)	% of WGA-pretreated Cells
Triton X-100 pellet	16.3 ± 4.1	6	25.0 ± 2.2	10
Triton X-100 supernatant	0.3 ± 0.2	1	7.7 ± 1.5	3
Triton X-100 pellet and supernatant	12.8 ± 5.8	5	63.3 ± 4.6	25

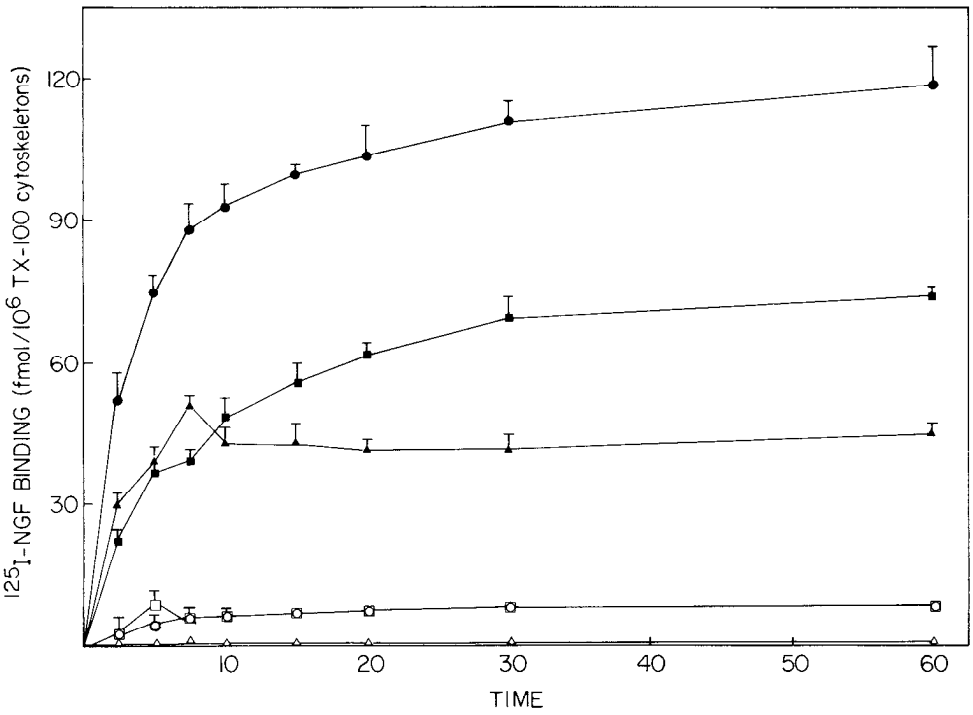

Figure 1. Time course of ^{125}I-NGF binding to Triton X-100 cytoskeletons. Triton X-100 cytoskeletons were prepared from PC12 cells (1.5 × 10^6/ml) which were previously treated with (*solid symbols*) or without (*open symbols*) WGA (50 μg/ml) for 30 min at 37°C. ^{125}I-NGF (610 pM) was added to Triton X-100 cytoskeletons, and at the indicated time total (●, ○), slowly dissociating (■, □), and rapidly dissociating (▲, △) binding were determined. *Error bars* indicate standard deviations.

letons prepared without WGA pretreatment, virtually all of the binding in this experiment was slowly dissociating. Binding to this receptor was again slower than that to the rapidly dissociating receptor in the presence of WGA. Thus, the time course of binding to the two receptor subtypes shows the same differences as those found in intact cells (Landreth and Shooter, 1980; Vale and Shooter, 1982). Since there is no plasma membrane, the time-dependent development of slowly dissociating binding in Triton X-100 cytoskeletons cannot be explained by internalization.

Triton X-100 extraction of cell surface proteins. Is the association of the NGF receptor with the Triton X-100 cytoskeleton induced by WGA unique, or are other membrane proteins affected in a similar fashion? To answer this question, we labeled cell surface proteins in a general manner with ^{125}I using a lactoperoxidase-catalyzed reaction and then examined the Triton X-100 solubility of these labeled proteins. Figure 2 shows that 33% of the ^{125}I-labeled cell surface proteins of PC12 cells remained associated with the cytoskeleton after Triton X-100 extraction. This value is more than 2-fold lower than the Triton X-100 insolubility of membrane proteins on cultured fibroblasts obtained by Ben Ze'ev et al. (1979). Despite the dramatic effects of WGA on the Triton X-100 solubility of the NGF receptor on PC12 cells, the lectin only increased by 7% the Triton X-100 insolubility of ^{125}I-labeled cell surface proteins. Con A, a lectin which recognizes mannose residues, had an effect similar to that of WGA.

Only a fraction of the total membrane-bound proteins contain carbohydrate moieties which will interact with WGA. Therefore, we investigated the Triton X-100 solubility of WGA receptors using an iodinated WGA derivative. ^{125}I-WGA (0.1 μg/ml) was added to PC12 cells along with increasing concentrations of unlabeled WGA, and samples were centrifuged through Triton X-100 extraction buffer to determine the detergent solubility of the bound ligand. Figure 3 shows that, with 0.1 μg/ml of ^{125}I-WGA, 80% of the cell-bound ^{125}I-WGA was solubilized by Triton X-100. However, increasing the receptor occupancy of WGA receptors by adding unlabeled WGA resulted in a decrease in the Triton X-100 solubility of the cell-bound tracer ^{125}I-WGA. Greater than 90% detergent insolubility was achieved at WGA concentrations in excess of 33 μg/ml. The NGF-receptor complex is converted to a Triton X-100-insoluble form at similar WGA concentrations (data not shown). This result indicates that most WGA-binding glycoproteins are unattached to the cytoskeleton but become associated with the Triton X-100 cytoskeleton by increasing the concentration of cell-bound WGA. The dependence upon WGA concentration for eliciting this effect suggests that this phenomenon may be the result of crosslinking cell surface proteins.

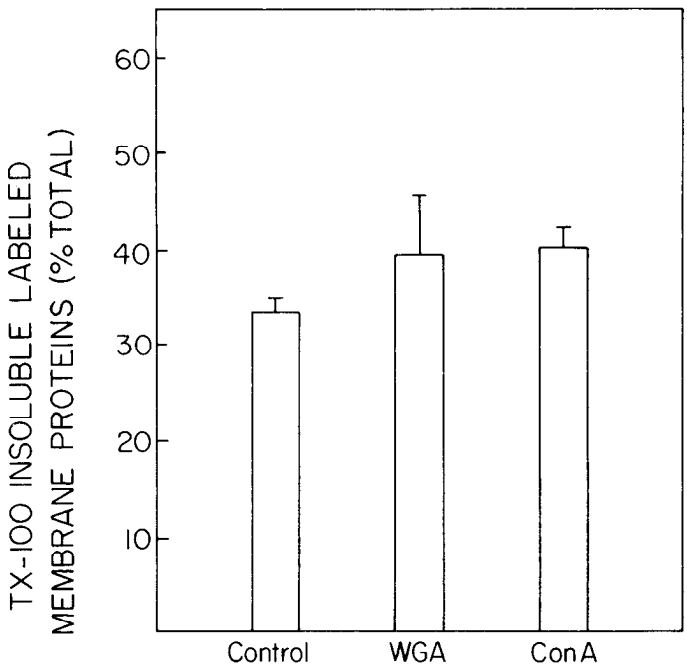

Figure 2. Triton X-100 solubility of lactoperoxidase-labeled cell surface proteins. PC12 cell surface proteins were labeled by lactoperoxidase as described under "Materials and Methods." Labeled cells (diluted to 0.5 × 10^6/ml) were incubated without lectins or with 50 μg/ml of either WGA or Con A for 30 min at 37°C. Cells were centrifuged (500 × *g*; 5 min), supernatants were removed, and cells were resuspended in Triton X-100 extraction buffer for 5 min at 4°C. Radioactivity associated with the Triton X-100 cytoskeleton was assayed by centrifugation through 0.15 M sucrose buffer as described for ^{125}I-NGF binding. Histograms show the percentage of radioactivity associated with the Triton X-100 cytoskeleton, and *error bars* indicate standard deviations.

The Triton X-100 solubility of WGA receptors and the conversion of NGF receptors to a slowly dissociating state exhibited similar WGA dose response curves (Fig. 3). At 10 μg/ml of WGA, 80% of NGF receptors were in a slowly dissociating state, and the same proportion of WGA receptors was also Triton X-100 insoluble. These results suggest that the Triton X-100 insolubility of WGA receptors after lectin binding may in some way be related to the change in the binding properties of the NGF receptor.

Composition of the Triton X-100 cytoskeleton. The morphology of the Triton X-100-insoluble material from PC12 cells was examined by scanning electron microscopy. Figure 4 shows a micrograph of PC12 cells, which have developed neurites after a 9-day exposure to NGF, after detergent extraction. Membrane structure was lost, and left behind was a nuclear remnant and an array of cytoskeletal filaments. Similar morphologies of Triton X-100 cytoskeletons are found with other cell types as well (Ben-Ze'ev et al., 1979; Burridge et al., 1982a, b). A latticework of filaments was also observed along the neurites and in the growth cones. Higher magnification views show that the cytoskeleton of detergent-extracted PC12 cells forms a discrete boundary at what was once the plasma membrane.

Approximately 65 to 75% of PC12 proteins were solubilized by Triton X-100. Some of the prominent proteins left behind in the Triton X-100-insoluble residue co-migrated in a polyarcylamide gel under denaturing and reducing conditions with the low molecular weight histones (M_r = 16,000 and 18,000), histone H1 (M_r = 31,000), actin (M_r = 43,000), tubulin (M_r = 55,000), myosin (M_r = 200,000), and some higher molecular weight proteins (M_r = 200,000) which may be actin-associated proteins (data not shown). WGA treatment of cells did not affect the protein pattern of the Triton X-100-insoluble residue.

Localization of NGF binding in the Triton X-100 cytoskeleton. Nuclear material is a prominent constituent of the Triton X-100 cytoskeleton and could play a role in the association of the receptor with detergent-insoluble material. To investigate this possibility, Triton X-100 solubility of NGF binding to enucleated PC12 cells was examined. Nuclei were removed from PC12 cells by cytochalasin B treatment followed by centrifugation. Cytoplasts derived by this procedure are viable for 1 to 2 days and are capable of extending neurites in response to NGF treatment (Nicholls et al., 1981). Figure 5 shows that cytoplasts bind ^{125}I-NGF, although to a lesser extent than intact cells. The lower level of binding is very likely due to partitioning of a portion of the plasma membrane to the nucleoplast as it pinches off during centrifugation. WGA converted ^{125}I-NGF binding to a slowly dissociating state both on cytoplasts and whole cells. Furthermore, 25% of the total binding to cytoplasts was Triton X-100 insoluble, and WGA increased this proportion to >80%. This result argues that the nucleus cannot be exclusively involved in the association of NGF receptors with Triton X-100-insoluble material.

The distribution of NGF receptors in the Triton X-100 cytoskeleton of differentiated PC12 cells can be visualized by light microscopic autoradiography. In this experiment, differentiated PC12 cells (treated with NGF for several days to induce neurites) were incubated with ^{125}I-NGF and co-treated with or without WGA. Intact cells treated this way were labeled to roughly the same extent regardless of the presence or absence of WGA (Fig. 6, *A* and *C*). However, when ^{125}I-NGF/WGA-treated cells were extracted with Triton X-100, heavy labeling over neurites and growth cones was retained (Fig. 6*D*). Conversely, when ^{125}I-NGF was presented to cells without the simultaneous addition of WGA, the resultant Triton X-100-extracted cell ghosts were only lightly labeled in perinuclear regions, with little binding retained on the processes or growth cones (Fig. 6*B*). With longer exposure times it was possible to detect some cytoskeletal binding on the neurites and growth cones, confirming both the existence of high affinity receptors in these regions (Carbenetto and Stach, 1972; M. J. Ignatius and E. M. Shooter, unpublished observations) and their association with the cytoskeleton, an association that is dramatically increased with WGA treatment.

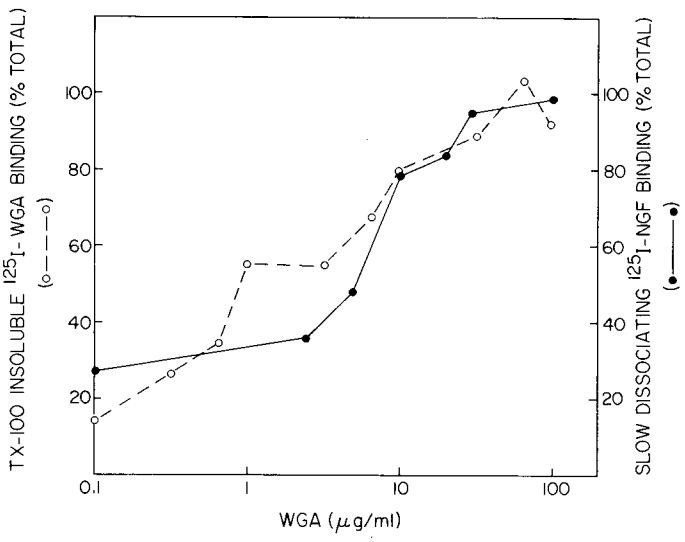

Figure 3. Concentration dependence of Triton X-100 insolubility of ^{125}I-WGA binding and WGA-induced NGF receptor conversion. In one experiment (O), tracer ^{125}I-WGA (0.1 μg/ml) was added to PC12 cells (10^6/ml) along with increasing concentrations of unlabeled WGA. Nonspecific ^{125}I-WGA binding was determined by adding 100 mM *N*-acetyl-D-glucosamine to the PBS/BSA/glucose solution. After a 45-min incubation at 37°C, total and Triton X-100-insoluble binding were assayed in the same manner described for ^{125}I-NGF binding. The percentage of Triton X-100-insoluble ^{125}I-WGA binding of the total is indicated over a range of WGA concentrations (combination of labeled and unlabeled ligand). In a separate experiment (●), ^{125}I-NGF (500 pM) was added to PC12 cells for 30 min at 37°C. WGA was then added at the indicated concentration for an additional 30 min prior to assaying total and slowly dissociating binding. The percentage of slowly dissociating ^{125}I-NGF binding of the total is expressed at each WGA concentration.

Discussion

NGF receptors exist in two different binding states. Covalent crosslinking of ^{125}I-NGF to PC12 cell surfaces (Hosang and Shooter, 1985) and selective trypsin sensitivity of binding (Landreth and Shooter, 1980; Schechter and Bothwell, 1981) indicate that slowly and rapidly dissociating bindings correlate with distinct structural or conformational receptor entities. This situation could arise with two discrete receptor proteins or a single protein capable of interconversion between two binding states. Evidence has been put forth in support of this latter hypothesis (Vale and Shooter, 1982, 1983; Buxser et al., 1983; Grob and Bothwell, 1983). From our results and those of Schechter and Bothwell (1981), one manifestation of the differences between the two receptor subtypes is their differential extraction by Triton X-100.

Many findings indicate that the correlation between slowly dissociating and Triton X-100-insoluble NGF binding is more than coincidental. Agents which increase the proportion of slowly dissociating binding such as WGA (Vale and Shooter, 1982; Buxser et al., 1983; Grob and Bothwell, 1983) and anti-NGF antibodies (Vale and Shooter, 1983) also enhance the resistance of binding to detergent solubilization. In the instance of WGA, time courses of the increase in slowly dissociating and Triton X-100-insoluble binding after lectin addition are superimposable (Vale and Shooter 1982). Recently, Jesaitis et al. (1984) have also observed that formation of a slowly dissociating complex between the N-formyl chemotactic peptide and its receptor on human granulocytes is accompanied by a change in the detergent solubility of the ligand-receptor complex. These investigators have also suggested that an association of the receptor with the cytoskeleton is involved in modulation of its affinity and possibly in its subsequent internalization.

Curiously, although present both in Triton X-100 cytoskeletons and whole cells, slowly dissociating binding is present in very low amounts in preparations of PC12 plasma membranes (Block and Bothwell, 1983). This result cannot be accounted for simply by receptor loss, since slowly dissociating binding can be regained by

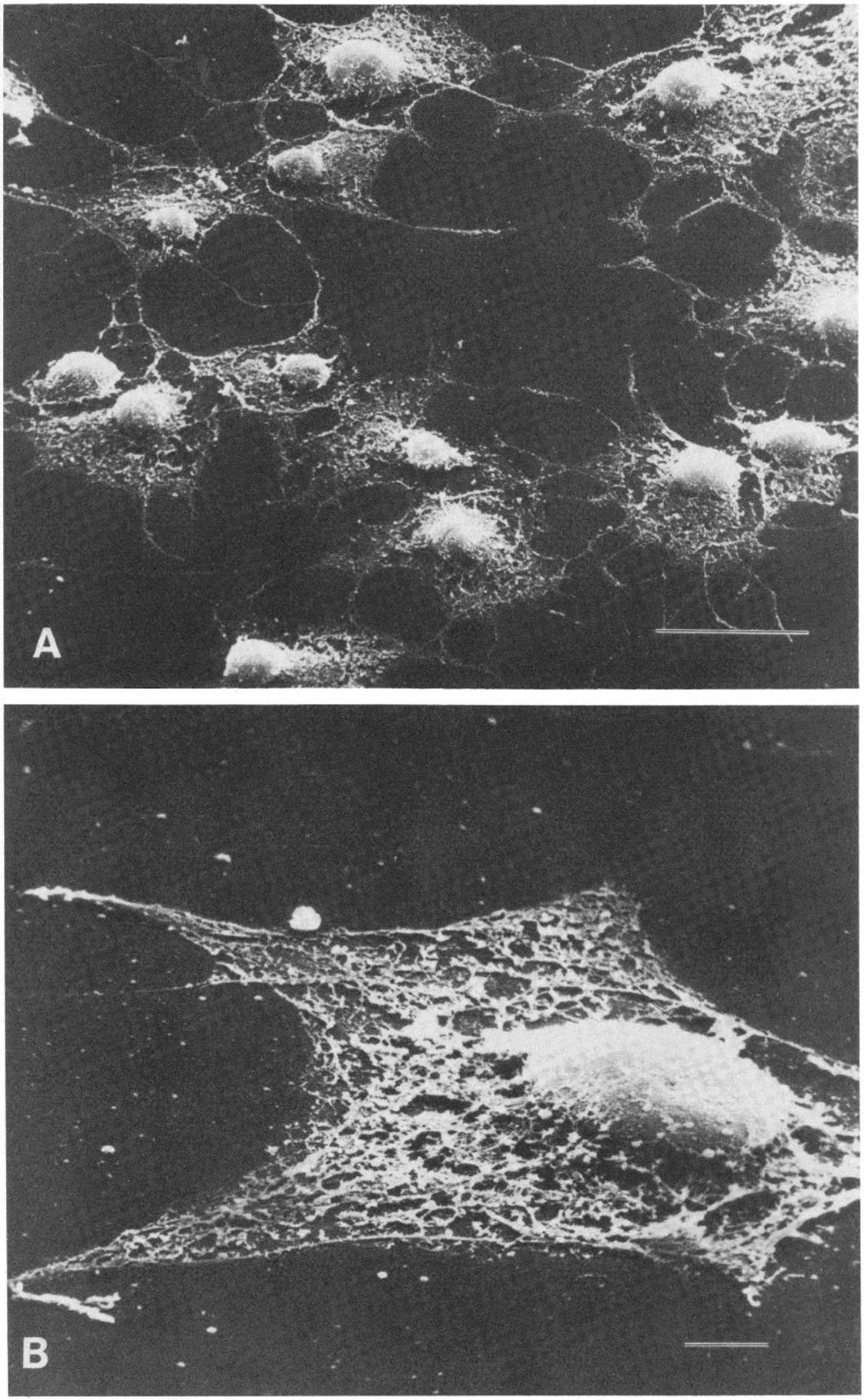

Figure 4. Scanning electron microscopy of PC12 Triton X-100 cytoskeletons. PC12 cells were grown on plastic coverslips and treated with NGF (50 ng/ml) for 5 days to induce neurites. Differentiated cells were washed twice with binding buffer and incubated with 0.5% Triton X-100 extraction buffer for 5 min at 4°C. Cytoskeletons were washed twice, fixed with 1% paraformaldehyde and 2% glutaraldehyde, and prepared for scanning electron microscopy as described under "Materials and Methods." All that remains of the cells after extraction are the nucleus, cytoskeleton, and a few Triton-resistant organelles. *Scale bars*: *A*, 10 μm; *B*, 0.1 μm.

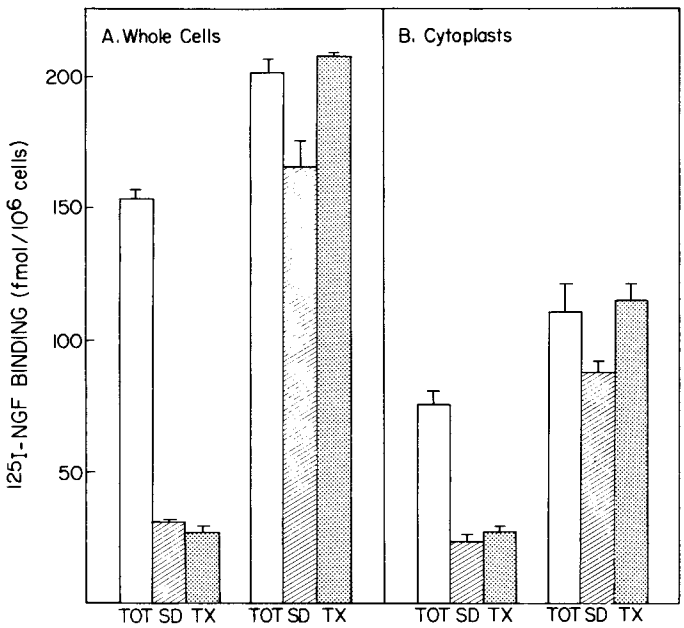

Figure 5. Effects of WGA on ^{125}I-NGF binding to enucleated cells. PC12 cytoplasts were obtained as described under "Materials and Methods." ^{125}I-NGF (1 nM) was added to cells (*A*) or cytoplasts (*B*) for 30 min at 37°C. Half of each sample then received WGA (50 μg/ml) for an additional 30 min prior to being assayed for total (*Tót*), slowly dissociating (*SD*), and Triton X-100-insoluble (*TX*) binding.

fusion of PC12 membranes with 3T3 cells which do not normally display NGF receptors (Block and Bothwell, 1983). Solubilized receptors also release bound NGF exclusively by rapidly dissociating kinetics (Buxser et al., 1983). These results imply that some element in whole cells or Triton X-100 cytoskeletons confers a high affinity, slowly dissociating state to the NGF receptor. A prominent candidate for performing this role is the cytoskeleton or a cytoskeleton-associated protein.

A number of membrane proteins have been reported to interact with cytoskeleton by various biochemical criteria (see Branton et al., 1981; Mescher et al., 1981; Pober et al., 1981; Carraway et al., 1983, for examples). Furthermore, an association between actin and intermediate filaments and the plasma membrane has been visualized by electron microscopy (Condeelis, 1979; Hubbard and Ma, 1983). At present, it is prudent to mention that evidence of a linkage between the NGF receptors and cytoskeletal proteins is circumstantial. We have shown that NGF receptors are present in a detergent-insoluble structure composed of cytoskeletal proteins and a nuclear remnant that allows the receptor to be pelleted at very low centrifugal forces. This association is also found in enucleated cells, suggesting that the nucleus is not solely responsible for this phenomenon. Also, NGF receptors can be induced with WGA to associate with the Triton X-100-insoluble residue corresponding to neurites and growth cones, regions which consist primarily of cytoskeletal elements. Furthermore, in preliminary results, we found that NGF receptors are partially released from the detergent-insoluble residue by agents which disrupt the cytoskeleton such as 0.4 M KCl, 10-sec sonication, and DNase I, an actin-depolymerizing agent. Cytochalasin B and colchicine had no effect, however. Nonetheless, the only way to convincingly demonstrate a linkage with the cytoskeleton is to document the co-sedimentation of the receptor with cytoskeletal proteins. In addition, our results do not rule out the possibility that the nucleus, in addition to the cytoskeleton, plays a role in the association of NGF receptor with the Triton X-100-insoluble residue, as has been previously proposed (Yankner and Shooter, 1979).

How is the interaction of the receptor and the cytoskeleton regulated? One possibility is that NGF may promote an association between the receptor and cytoskeleton. In support of this notion, we found that Triton X-100 insolubility of the receptor is greater when it is occupied with NGF before extraction (Table I). Conversely, WGA treatment of cells causes a maximal association of the receptor with the cytoskeleton whether or not NGF is present prior to detergent extraction. Modulation of the association of membrane proteins with the cytoskeleton by lectins has also been reported by several investigators. Actin and myosin have been localized under lectin-induced patches and caps of membrane glycoproteins in lymphocytes (Bourguignon and Singer, 1977) and Dictyostelium (Condeelis, 1979). Also, Con A-induced interactions between two platelet glycoproteins (Painter and Ginsberg, 1982) and three neutrophil cell surface proteins (Sheterline and Hopkins, 1981) and the cytoskeleton have been documented. Lectin-induced crosslinking of proteins appears important for this effect, since a succinylated Con A derivative which is functionally monovalent does not change substantially the detergent solubility of such proteins. Antibody-induced cross-bridging of cell surface proteins such as surface IgG on lymphocytes (Flanagan and Koch, 1978; Woda and McFadden, 1983) also induces cytoskeletal attachment. We have observed a similar phenomenon with anti-NGF antibodies which caused NGF-receptor complexes to co-sediment with the Triton X-100-insoluble residue (Vale and Shooter, 1983). This response was due to antibody-induced crosslinking since monovalent Fab fragment had no effect.

The mechanism by which crosslinking of cell surface proteins causes their association with the cytoskeleton is unclear. Binding of a lectin to certain glycoproteins could cause a conformational change sufficient to induce a direct transmembrane interaction with the cytoskeleton; however, our results show that all receptors for WGA, not a subset, are rendered insoluble to Triton X-100 in a manner dependent upon the concentration of lectin added. This finding implies some sort of cooperative interaction between occupied WGA receptors which makes them detergent insoluble. Studies which observed attachments of only certain glycoproteins with the cytoskeleton by Con A may have been limited by the ability to detect only the most abundant lectin receptors (Sheterline and Hopkins, 1981; Painter and Ginsberg, 1982).

Three explanations are consistent with the results we have obtained. The first is that the restriction of lateral mobility of glycoproteins by lectin-induced crosslinking (Schlessinger et al., 1977; Koppel et al., 1981) enhances their interaction with the underlying cytoskeletal network. An alternative possibility is that patches of crosslinked membrane proteins produce strong lipid-protein interactions sufficient to prevent detergent extraction of these membrane domains. In support of this view, Condeelis (1979) found that membrane in regions containing clustered Con A-receptor complexes is more resistant to disruption by Triton X-100. These areas also contain large amounts of attached actin and myosin which may contribute to the association of Con A receptors with detergent-insoluble material. The third hypothesis, which we favor, is that a subset (<20%) of the lectin receptors is associated with the cytoskeleton and that lectin addition serves to crosslink these proteins to others that are normally unrestrained in the lipid bilayer, thereby making all occupied lectin-binding proteins Triton X-100 insoluble. A similar conclusion concerning the mechanism of Con A-induced attachment of glycoproteins to the cytoskeleton was reached by Jung et al. (1984), who showed that Con A crossbridged unattached glycoproteins to a glycoprotein which is strongly associated with the submembrane cytoskeleton.

An interaction of the NGF receptor with the cytoskeleton could have several important consequences for the action of this hormone. Although the mechanism by which NGF affects the differentiated state of responsive cells is unknown, dose response curves indicate that these effects are mediated by high affinity, slowly dissociating receptors (Sutter et al., 1979b). NGF also influences the cytoskeletal organization of PC12. Within 30 sec after addition, NGF induces prominent cell surface ruffling (Connolly et al., 1979), whereas at later times, reorganization of microfilaments and microtubules oc-

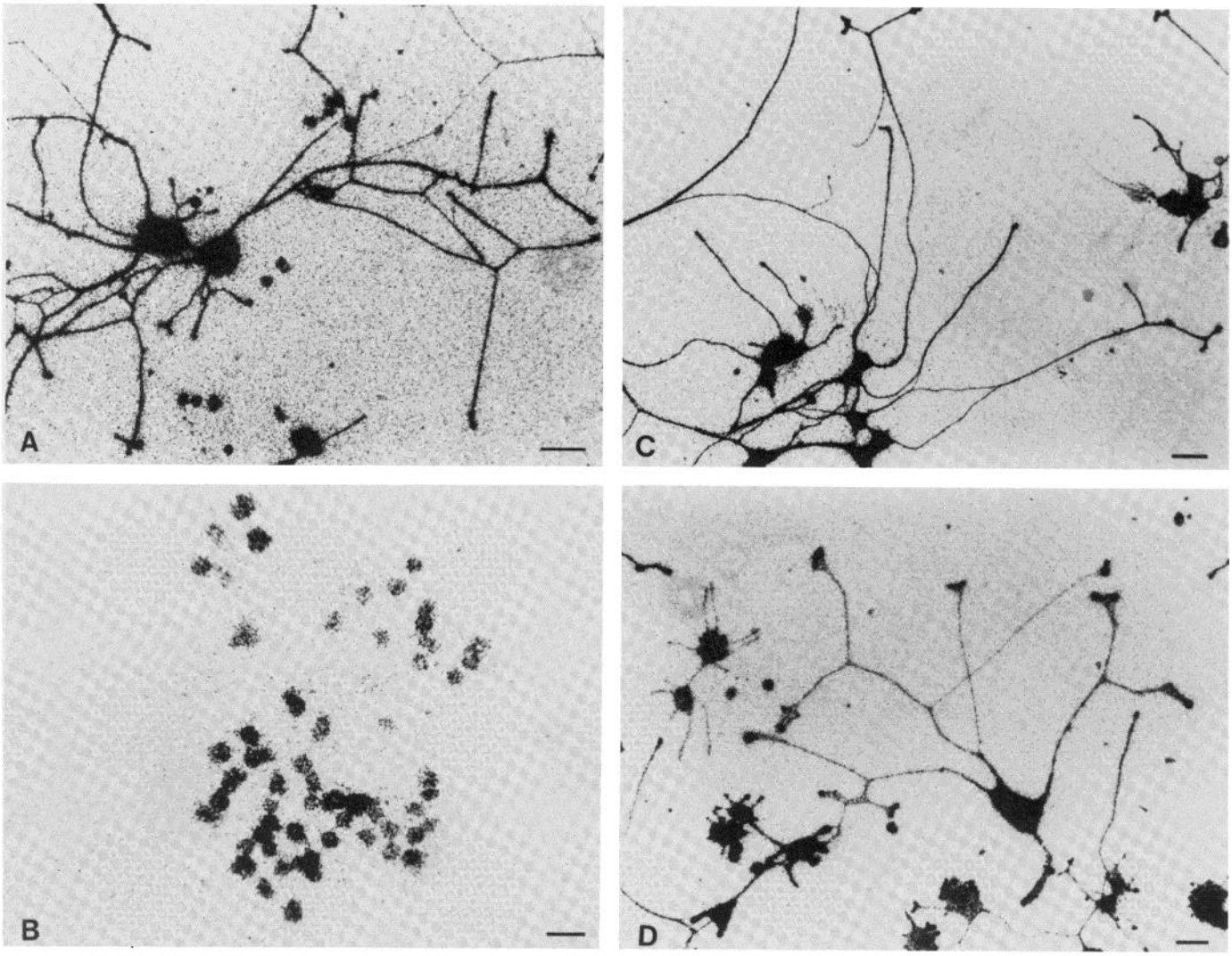

Figure 6. Autoradiography of Triton X-100-insoluble ^{125}I-NGF binding to differentiated PC12 cells. Cells were grown for 8 days in NGF. On day 9 cells were washed three times for 1 hr with NGF-free medium; then, ^{125}I-NGF (1 nM) was added in the presence or absence of WGA (50 μg/ml) for 30 min at 37°C. The cells were then either left intact or extracted in 0.5% Triton X-100 extraction buffer for 5 min at 4°C and subsequently washed. (See scanning electron micrographs of Fig. 4, for structures that remain after identical treatment.) After fixation and processing for autoradiography (see "Materials and Methods"), the Triton X-100 cytoskeletons were photographed with brightfield optics. Treatments were as follows: *A*, ^{125}I-NGF alone; *B*, ^{125}I-NGF and Triton X-100, *C*, ^{125}I-NGF and WGA; and *D*, ^{125}I-NGF, WGA, and Triton X-100. WGA has no effect on the total labeling seen on intact cells (*A* and *C*), whereas on extracted cells (*B* and *D*) WGA pretreatment results in the retention of heavy labeling over the cytoskeletal-rich neurites and growth cones (*D*) compared to only minimal perinuclear labeling seen without WGA (*B*). *Scale bars*, 10 μm.

curs, culminating in the outgrowth of neurites. Furthermore, binding of NGF to receptors at growth cones redirects the orientation of these structures in a chemotactic fashion even in the absence of a connection with the cell body (Gundersen and Barrett, 1979; Seeley and Greene, 1983). Interaction between slowly dissociating receptors and the cytoskeleton may be involved in mediating these responses.

Furthermore, NGF receptors undergo cell surface clustering and subsequent internalization (Levi et al., 1980), and several studies indicate that the slowly dissociating receptor is the species which becomes internalized (Yankner and Shooter, 1982; Bernd and Greene, 1983). Attachments between cell surface proteins and cytoskeletal filaments have been implicated in the internalization process (Salisbury et al., 1980). To obtain more detailed knowledge of the differences between rapidly and slowly dissociating receptors and their possible interactions with the subcortical cytoskeleton, it will be necessary to reconstitute such functions or interactions with isolated membranes or purified receptors (Puma et al., 1983) in liposomes. Such experiments may help in defining some of the initial events in the mechanism of action of this hormone.

References

Ben-Ze'ev, A., A. Duerr, F. Solomon, and S. Penman (1979) The outer boundary of the cytoskeleton: A lamina derived from plasma membrane proteins. Cell *17:* 859–865.

Bennett, V. (1982) The molecular basis for membrane-cytoskeleton association in human erythrocytes. J. Cell. Biochem. *18:* 49–65.

Bernd, P. S., and L. A. Greene (1983) Association of ^{125}I-NGF with PC12 pheochromocytoma cells: Evidence for internalization via high-affinity receptors only and for long-term regulation by NGF of both high- and low-affinity receptors. Soc. Neurosci. Abstr. *9:* 842.

Block, T., and M. Bothwell (1983) The nerve growth factor receptor on PC12 cells: Interconversion between two forms with different binding properties. J. Neurochem. *40:* 1654–1663.

Bourguignon, L. Y. W., and S. J. Singer (1977) Transmembrane interactions and the mechanism of capping of surface receptors by their specific ligands. Proc. Natl. Acad. Sci. U. S. A. *74:* 5031–5035.

Branton, D., C. M. Cohen, and J. Tyler (1981) Interaction of cytoskeletal proteins on the human erythrocyte membrane. Cell *24:* 24–32.

Bray, D., and D. Gilbert (1981) Cytoskeletal elements in neurons. Annu. Rev. Neurosci. *4:* 505–523.

Brown, S., W. Levinson, and J. A. Spudich (1976) Cytoskeletal elements of chick embryo fibroblasts revealed by detergent extraction. J. Supramol. Struct. *5:* 119–130.

Burridge, K., T. Kelley, and L. Connell (1982a) Proteins involved in the attachment of actin to the plasma membrane. Philos. Trans. R. Soc. Lond. (Biol.) *299:* 291–299.

Burridge, K., T. Kelley, and P. Mangeat (1982b) Nonerythrocyte spectrins: Actin-membrane attachment proteins occurring in many cell types. J. Cell Biol. *95:* 478–486.

Buxser, S. E., D. J. Kelleher, L. Watson, P. Puma, and G. L. Johnson (1983) Change in state of nerve growth factor receptor. Modulation of receptor affinity by wheat germ agglutinin. J. Biol. Chem. *258:* 3741–3749.

Carbonetto, S. T., and R. W. Stach (1980) Localization of nerve growth factor bound to cultured sensory neurons. Soc. Neurosci. Abstr. *6:* 375.

Carraway, C. A. C., G. Jung, and K. L. Carraway (1983) Isolation of actin-containing transmembrane complexes from ascites adenocarcinoma sublines having mobile and immobile receptors. Proc. Natl. Acad. Sci. U. S. A. *80:* 430–434.

Condeelis, J. (1979) Isolation of concanavalin A caps during various stages of formation and their association with actin and myosin. J. Cell Biol. *80:* 751–758.

Connolly, J. L., L. A. Greene, R. R. Viscarello, and W. D. Riley (1979) Rapid, sequential changes in surface morphology of PC12 pheochromocytoma cells in response to nerve growth factor. J. Cell Biol. *82:* 820–827.

DeBault, C. E. (1973) A critical point drying technique for SEM of tissue culture cells grown on plastic substratum. Illinois Institute of Technology Research Institute (IITRI)/SEM: 317–324.

Flanagan, J., and G. L. E. Koch (1978) Cross-linked surface Ig attaches to actin. Nature *273:* 278–281.

Greene, L. A., and A. S. Tischler (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. U. S. A. *73:* 2424–2428.

Grob, P. M., and M. A. Bothwell (1983) Modification of nerve growth factor receptor properties by wheat germ agglutinin. J. Biol. Chem. *258:* 14136–14143.

Gundersen, R. W., and J. N. Barrett (1979) Neuronal chemotaxis: Chick dorsal-root axons turn toward high concentrations of nerve growth factor. Science *206:* 1079–1080.

Hellewell, S. B., and D. L. Taylor (1979) The contractile basis of ameboid movement. VI. The solation-contraction hypothesis. J. Cell Biol. *83:* 633–648.

Herrup, K., and H. Thoenen (1979) Properties of the nerve growth factor receptor of a clonal line of rat pheochromocytoma (PC12) cells. Exp. Cell Res. *121:* 71–78.

Hosang, M., and E. M. Shooter (1985) Molecular characteristics of nerve growth factor receptors in PC12 cells. J. Biol. Chem. *260:* 655–662.

Hubbard, A. L., and A. Ma (1983) Isolation of rat hepatocyte plasma membranes. II. Identification of membrane-associated cytoskeletal proteins. J. Cell Biol. *96:* 230–239.

Hynes, R. O. (1973) Alteration of cell-surface proteins by viral transformation and by proteolysis. Proc. Natl. Acad. Sci. U. S. A. *70:* 3170–3174.

Jesaitis, A. J., J. R. Naemura, L. A. Sklar, C. G. Cochrane, and R. G. Painter (1984) Rapid modulation of N-formyl chemotactic peptide receptors on the surface of human granulocytes: Formation of slowly dissociating ligand-receptor complexes in transient association with cell cytoskeleton. J. Cell Biol. *98:* 1378–1387.

Jung, G., R. M. Helm, C. A. C. Carraway, and K. L. Carraway (1984) Mechanism of concanavalin A-induced anchorage of the major cell surface glycoproteins to the submembrane cytoskeleton in 13762 ascites mammary adenocarcinoma cells. J. Cell Biol. *98:* 179–187.

Koppel, D. E., M. P. Sheetz, and M. Schindler (1981) Matrix control of protein diffusion in biological membranes. Proc. Natl. Acad. Sci. U. S. A. *78:* 3576–3580.

Kopriwa, B. M., and L. P. Leblond (1962) Improvement in the coating technique of radioautography. J. Histochem. Cytochem. *10:* 269–284.

Landreth, G. E., and E. M. Shooter (1980) Nerve growth factor receptors in PC12 cells: Ligand-induced conversion from low to high affinity states. Proc. Natl. Acad. Sci. U. S. A. *77:* 4751–4755.

Levi, A., Y. Shechter, E. J. Neufeld, and J. Schlessinger (1980) Mobility, clustering and transport of nerve growth factor in embryonal sensory cells and in a sympathetic neuronal cell line. Proc. Natl. Acad. Sci. U. S. A. *77:* 3469–3473.

Mescher, M. F., M. J. L. Jose, and S. P. Balk (1981) Actin-containing matrix associated with the plasma membrane of murine tumor and lymphoid cells. Nature *289:* 139–144.

Mobley, W. C., A. Schenker, and E. M. Shooter (1976) Characterization and isolation of proteolytically modified nerve growth factor. Biochemistry *15:* 5543–5551.

Nicholls, R., C. E. Chandler, and E. M. Shooter (1981) Enucleation of PC12 cells. Soc. Neurosci. Abstr. *7:* 150.

Painter, R. G., and M. Ginsberg (1982) Concanavalin A induces interactions between surface glycoproteins and the platelet cytoskeleton. J. Cell Biol. *92:* 565–573.

Pober, J. S., B. C. Guild, J. L. Strominger, and W. R. Veatch (1981) Purification of HLA-A2 antigen, fluorescent labeling of its intracellular region, and demonstration of an interaction between fluorescently labeled HLA-A2 antigen and lymphoblastoid cell cytoskeleton proteins *in vitro*. Biochemistry *20:* 5625–5633.

Prives, J., A. B. Fulton, S. Penman, M. P. Daniels, and C. N. Christian (1982) Interaction of the cytoskeletal framework with acetylcholine receptor on the surface of embryonic muscle cells in culture. J. Cell Biol. *92:* 231–236.

Puma, P., S. E. Buxser, L. Watson, D. J. Kelleher, and G. L. Johnson (1983) Purification of the receptor for nerve growth factor from A875 melanoma cells by affinity chromatography. J. Biol. Chem. *258:* 3370–3375.

Sahyoan, N. E., H. Le Vine III, J. Davis, G. M. Hebdon, and P. Cuatrecasas (1981) Molecular complexes involved in the regulation of adenylate cyclase. Proc. Natl. Acad. Sci. U. S. A. *78:* 6158–6162.

Salisbury, J. L., J. S. Condeelis, and P. Satir (1980) Role of coated vesicles, microfilaments, and calmodulin in receptor-mediated endocytosis by cultured B lymphoblastoid cells. J. Cell Biol. *87:* 132–141.

Schechter, A. L., and M. A. Bothwell (1981) Nerve growth factor receptors on PC12 cells: Evidence for two receptor classes with differing cytoskeletal association. Cell *24:* 867–874.

Schlessinger, J., D. Axelrod, D. E. Koppel, W. W. Webb, and E. L. Elson (1976) Lateral transport of a lipid probe and labeled proteins on a cell membrane. Science *195:* 307–309.

Schlessinger, J., E. L. Elson, W. W. Webb, I. Yahara, U. Rutishauser, and G. M. Edelman (1977) Receptor diffusion of cell surfaces modulated by locally bound concanavalin A. Proc. Natl. Acad. Sci. U. S. A. *74:* 1110–1114.

Seeley, P. J., and L. A. Greene (1983) Short-latency local actions of nerve growth factor at the growth cone. Proc. Natl. Acad. Sci. U. S. A. *80:* 2789–2793.

Sheterline, P., and C. R. Hopkins (1981) Transmembrane linkage between surface glycoproteins and components of the cytoplasm in neutrophils. J. Cell Biol. *90:* 743–754.

Stossel, T. P. (1982) The structure of cortical cytoplasm. Philos. Trans. R. Soc. lond. (Biol.) *299:* 275–289.

Sutter, A., R. J. Riopelle, R. M. Harris-Warrick, and E. M. Shooter (1979a) Nerve growth factor receptors. Characterization of two distinct classes of binding sites on chick embryo sensory ganglia cells. J. Biol. Chem. *254:* 5972–5982.

Sutter, A., R. J. Riopelle, R. M. Harris-Warrick, and E. M. Shooter (1979b) The heterogeneity of nerve growth factor receptors. In *Transmembrane Signaling*, M. Bitensky, R. J. Collier, D. F. Steiner, and C. F. Fox, eds., pp. 659–667, Alan Liss Inc., New York.

Thoenen, H., and Y. A. Barde (1980) Physiology of nerve growth factor. Physiol. Rev. *60:* 1284–1334.

Vale, R. D., and E. M. Shooter (1982) Alteration of binding properties and cytoskeletal attachment of nerve growth factor receptors in PC12 cells by wheat germ agglutinin. J. Cell Biol. *94:* 710–717.

Vale, R. D., and E. M. Shooter (1983) Conversion of nerve growth factor-receptor complexes to a slowly dissociating, Triton X-100 insoluble state by anti nerve growth factor antibodies. Biochemistry *22:* 5022–5028.

Woda, B. A., and M. L. McFadden (1983) Association of lymphocyte membrane proteins with the detergent-insoluble cytoskeletal matrix. Fed. Proc. *42:* 321.

Yankner, B. A., and E. M. Shooter (1979) Nerve growth factor in the nucleus: Interaction with receptors on the nuclear membrane. Proc. Natl. Acad. Sci. U. S. A. *76:* 1269–1273.

Yankner, B. A., and E. M. Shooter (1982) The biology and mechanism of action of nerve growth factor. Annu. Rev. Biochem. *51:* 845–868.