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IDENTIFICATION OF THE INSULIN RECEPTOR IN UNDIFFERENTIATED AND DIFFERENTIATED NB-15 MOUSE NEUROBLASTOMA CELLS BY AFFINITY LABELING

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Plasma membranes prepared from clonal NB-15 mouse neuroblastoma cells were sequentially incubated with ^{125}I-labeled insulin (10 nM) and the bifunctional cross-linking agent disuccinimidyl suberate. This treatment resulted in the cross-linking of ^{125}I-labeled insulin to a polypeptide that gave an apparent M_r of 135000 on a sodium dodecyl sulfate-polyacrylamide gel electrophoresed in the presence of 10% β-mercaptoethanol. Affinity labeling of this polypeptide was inhibited by the presence of 5 μM unlabeled insulin, but not by 1 μM unlabeled nerve growth factor. Using the same affinity labeling technique, ^{125}I-labeled nerve growth factor (1 nM) did not label any polypeptide appreciably in the plasma membranes of NB-15 cells but labeled an M_r 145000 and an M_r 115000 species in PC-12 rat pheochromocytoma cells. The number of insulin binding sites per cell in the intact differentiated NB-15 mouse neuroblastoma cells was approx. 6-fold greater than that in the undifferentiated NB-15 mouse neuroblastoma cells as measured by specific binding assay, suggesting an increase of the number of insulin receptors in NB-15 mouse neuroblastoma cells during differentiation.

Introduction

Mouse neuroblastoma cells derived from a spontaneous tumor of the neural crest in mice generally remain in a relatively immature state of differentiation [1,2]. Nevertheless, in the presence of cAMP analogues or agents which increase cellular cAMP content, these cells can undergo differentiation, a process defined by the morphological appearance of long neurites and biochemical expression of neurotransmitter-metabolizing enzymes [3,4]. Since the differentiated mouse neuroblastoma cells exhibit many of the properties of mature neurons, they have been used extensively as a model to study neuronal development [2,4].

Bottenstein and Sato [5] have developed a serum-free hormone-supplemented growth medium (called N2 medium in Ref. 5) to grow the rat neuroblastoma B104 cells. Spoerri et al. [6] have subsequently shown that mouse neuroblastoma cells (clone Neuro-2a) also proliferate in this medium. One essential hormone supplemented in this medium is insulin, suggesting that insulin has a physiological role in maintaining the growth and survival of these cells in a serum-free medium. It is generally recognized that the first step in the action of polypeptide hormones on target cells is binding to a membrane receptor [7]. The fact that insulin is essential for the proliferation of rat and

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Abbreviation: PMSF, phenylmethylsulfonyl fluoride.

mouse neuroblastoma cells maintained in a serum-free medium implies that insulin receptors or insulin-like growth factor receptors may be present in these cells.

In order to demonstrate unequivocally the presence of insulin receptors on the plasma membrane of mouse neuroblastoma cells, we have used the affinity labeling technique to probe the insulin effector system of NB-15 mouse neuroblastoma cells. Using this technique together with sodium dodecyl sulfate gel electrophoresis, we showed here that ^{125}I-labeled insulin specifically and covalently linked to a polypeptide that gave an apparent M_r of 135 000 in the plasma membranes of NB-15 mouse neuroblastoma cells. Specific equilibrium binding assay carried out with intact NB-15 mouse neuroblastoma cells indicated a 6-fold increase of insulin binding sites in NB-15 cells after cell differentiation. Using the same affinity labeling technique, we were able to identify nerve growth factor receptor protein in PC-12 rat pheochromocytoma cells, but not in NB-15 mouse neuroblastoma cells.

Materials and Methods

Materials. Na^{125}I (17 Ci/mg) was purchased from New England Nuclear, Boston, MA. Nerve growth factor (2.5 S) was purchased from Collaborative Research, Waltham, MA. Sephadex G-25-50, chloramine T, porcine pancreatic insulin, Bacitracin, phenylmethylsulfonyl fluoride (PMSF), and lactoperoxidase (EC 1.11.1.7) were obtained from Sigma, St Louis, MO. Hydroxysuccinimidyl-*p*-azidobenzoate, disuccinimidyl suberate and iodobeads were obtained from Pierce Co., Rockford, IL. All the tissue culture supplies came from Gibco, Grand Island, NY. PC-12 cells were gifts from Dr. L.A. Greene, New York Medical School, and Dr. M. Bothwell, Princeton University.

Cells. The clonal line NB-15 of mouse neuroblastoma was maintained in Dulbecco's Modified Eagle's medium supplemented with 10% fetal calf serum at 37°C in a Forma water-jacketed CO_2 incubator (95% air/5% CO_2, water-saturated atmosphere) [8]. The clonal line PC-12 of rat pheochromocytoma was maintained in Dulbecco's medium supplemented with 5% fetal calf serum and 10% horse serum. Cells were used in their late log phase of growth unless otherwise indicated. Differentiation of NB-15 mouse neuroblastoma cells was induced by the addition of 1 mM dibutyryl cyclic AMP and 0.5 μM 3-isobutyl-1-methylxanthine to the culture, 15 h after plating, as previously described [8].

Preparation of membranes. Plasma membranes from NB-15 cells and PC-12 cells were prepared by hypotonic disruption and two-step discontinuous sucrose gradient centrifugation as previously described [9]. The purified plasma membranes exhibited an increase of specific activity of alkaline phosphatase by 5–8-fold.

Iodination of insulin and nerve growth factor. The hormones were radiolabeled with ^{125}I by using a modification of the stoichiometric chloramine T method of Roth [10] or the lactoperoxidase catalyzed iodination method [11]. Briefly, 5 μg of hormone in 5 μl 1 mM HCl was added to 1 mCi ^{125}I in 0.1 M NaOH. An aliquot of 20 μl of NaH_2PO_4 (0.2 M) was added to neutralize the NaOH, and the reaction was started by the addition of 5 μl of chloramine T (15 μg/ml) or by 20 μg lactoperoxidase and 2 μl of 4.4 mM H_2O_2. The reaction was stopped by adding 5 μl of sodium bisulfite (30 μg/ml). The labeled hormones were removed from the reaction vessel with another 100 μl of phosphate buffer (pH 7.2) containing 1% bovine serum albumin and purified by column chromatography over Sephadex G-25-50. More than 90% of the purified material was trichloroacetic acid precipitable and had specific activities of approx. 50 Ci/g protein.

Affinity labeling. Plasma membrane fractions containing approx. 200 μg protein were incubated with $1 \cdot 10^{-8}$ M ^{125}I-labeled insulin (25–50 Ci/g) or 1 nM ^{125}I-labeled nerve growth factor (25–50 Ci/g) in a 200 μl buffer containing 1 mM PMSF, 1.5 mM Bacitracin, 10 mg/ml bovine serum albumin, 1 mM EDTA, 1.2 mM $MgCl_2$, 5 mM KCl, 140 mM NaCl and 100 mM NaH_2PO_4/Na_2HPO_4 (pH 7.7) for 60 min at room temperature. The cross-linking agents disuccinimidyl suberate, freshly dissolved in dimethyl sulfoxide, was added to a final concentration of 0.2 mM. The reaction was carried out at 4°C for 5 min and terminated by an addition of 2 ml cold 100 mM Tris-HCl (pH 7.5). The membrane fractions were centrifuged at 25 000 × *g* for 20 min and dissolved in a buffer containing 2% sodium dodecyl sulfate and 50 mM

Tris-HCl (pH 6.8) with or without reductant β-mercaptoethanol or dithiothreitol. Samples were boiled for 3 min and electrophoresed on the discontinuous system of Laemmli [12], using 7.5% polyacrylamide slab gels, unless otherwise indicated. Autoradiograms were made from the dried gel on Kodak X-ray film with enhancing screen.

Insulin binding assay. Monolayer cultures were washed once with cold phosphate-buffered saline and harvested by centrifugation at $500 \times g$ for 5 min. The cells were further washed twice with cold phosphate-buffered saline and resuspended in a phosphate buffer containing 100 mM phosphate (pH 7.7), 140 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1 mM EDTA, 10 mg/ml bovine serum albumin, 1 mM PMSF and 1.5 mM Bacitracin, an inhibitor of insulin degradation. Binding assays were carried out in a final volume of 0.30 ml for 20 min at 17°C in the presence of ^{125}I-labeled insulin. Preliminary studies indicated that maximal binding of insulin in mouse neuroblastoma cells occurred 20–30 min after the initiation of incubation at 17°C (data not shown), similar to that observed by Olefsky and Kao in IM-9 lymphocytes [13]. Cell densities and concentrations of ^{125}I-labeled insulin are indicated in the text and legend to Fig. 3. After incubation, aliquots (75 μl) of the cell suspension were layered over a two-step gradient of 0.15 and 0.35 M sucrose in phosphate-buffered saline in a 0.5-ml microfuge tube. The tube was immediately centrifuged at 12000 rpm for 3 min at 5°C. The microfuge tubes were frozen immediately in a dry ice/acetone bath, and the tips of microfuge tubes containing the cell pellets were cut off and counted in an Beckman LS 7000 liquid scintillation counter. Duplicate tubes containing ^{125}I-labeled insulin and 5 μM nonradioactive insulin were assayed in parallel to determine nonspecific binding, which accounted for 30–40% of the total binding.

Results

Purified plasma membranes of NB-15 cells were affinity labeled with $1 \cdot 10^{-8}$ M ^{125}I-labeled insulin, using a cross-linking agent, disuccinimidyl suberate. Electrophoresis of the affinity-labeled membranes on sodium dodecyl sulfate-polyacrylamide slab gels in the presence of 10% β-mercaptoethanol and autoradiography of these gels revealed a prominently labeled band migrating at a position corresponding to M_r 135000 (Fig. 1, lane B). This band was not radiolabeled in the absence of cross-linking agent (Fig. 1, lane A) or in the presence of 5 μM unlabeled insulin (Fig. 1, lane C). In the complete absence of reductant (Fig. 1, lane D), much of the labeled material stayed at the top of the 7.5% gel and was not properly resolved. The addition of 5 mM dithiothreitol led to the appearance of a prominently labeled band at M_r 135000 and a faintly labeled band at M_r 350000 (Fig. 1, lane E). These data indicated that: (i) ^{125}I-labeled insulin could be specifically cross-linked to a membrane-bound polypeptide in mouse neuroblastoma cells to form an adduct with an apparent M_r of 135000; (ii) the M_r 135000 polypeptides were associated via disulfide linkage to form high-molecular-weight species, M_r 350000 or

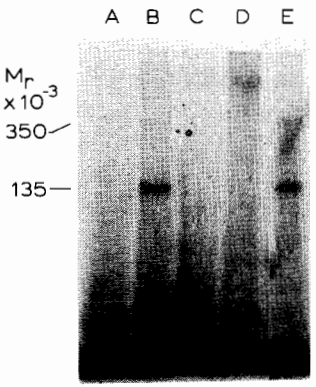

Fig. 1. Autoradiogram of a sodium dodecyl sulfate-polyacrylamide gel electrophoretic pattern of NB-15 mouse neuroblastoma membranes cross-linked to ^{125}I-labeled insulin. During the cross-linking reaction, the membrane samples in all lanes were incubated with 10 nM ^{125}I-labeled insulin in the presence (lanes B–E) or in the absence (lane A) of disuccinimidyl suberate (0.2 mM). Membrane sample in lane C was also incubated with 5 μM unlabeled insulin prior to cross-linking. Samples of affinity-labeled membranes were solubilized in the presence of 2% sodium dodecyl sulfate with 10% β-mercaptoethanol (lanes A, B and C) or with 5 mM dithiothreitol (lane E) or without any reductant (lane D). Samples were then electrophoresed on a 7.5% polyacrylamide slab gel. Protein standards used were: myosin (M_r 200000), β-galactosidase (M_r 130000), phosphorylase b (M_r 94000) bovine serum albumin (M_r 67000), catalase (58000), ovalbumin (45000), aldolase (40000) and chymotrypsinogen (25000). Each lane contained membranes equivalent to 75 μg proteins.

higher, in the absence of reductant. Extensive studies of insulin receptors using the same cross-linking technique on rat adipose cells [14,15] and rat liver cells [16] have led Czech and co-workers to propose that the insulin receptor in these cells is a symmetric disulfide-linked heterotetramer composed of two α- (M_r 125 000) and two β- (M_r 90 000) glycoprotein subunits in the configuration (β-S-S-α)-S-S-(α-S-S-β) [17]. The absence of a detectable band in the M_r 90 000 region on the autoradiograph in Fig. 1 suggested either no or very little labeling of the β-subunit of insulin receptors under these experimental conditions. Poor labeling of the β-subunit as compared to the predominant labeling of the α-subunit of insulin receptors in other cell types has been previously reported [18,19]. The specificity of the labeling of the M_r 135 000 band in NB-15 cells (Fig. 1) and the similarity in apparent molecular weight between this labeled band and the α-subunit of known insulin receptor in other cell types suggest the presence of insulin receptors on NB-15 mouse neuroblastoma cells.

The presence of insulin receptors on NB-15 mouse neuroblastoma cells implies that insulin may have some physiological function in these cells. Indeed we have found that insulin at 10^{-8}–10^{-9} M potentiated the asparagin-mediated induction of ornithine decarboxylase activity in NB-15 neuroblastoma cells; the degree of potentiation was more pronounced in the differentiated than in the undifferentiated NB-15 cells (Chen and Rinehart, unpublished data). This observation prompted us to compare the insulin binding in the undifferentiated and differentiated NB-15 mouse neuroblastoma cells using (i) affinity labeling technique and (ii) equilibrium ^{125}I-labeled insulin binding assay. When equal amounts of plasma membranes prepared from undifferentiated and differentiated NB-15 cells were cross-linked to ^{125}I-labeled insulin and analyzed according to the procedures described in Material and Methods, a single protein band at M_r 135 000 was labeled specifically in both cell types (Fig. 2 lanes A, B and D, E). The labeling intensity of the M_r 135 000 band in the differentiated cells was 1.5-fold more intense than that in the undifferentiated cells (Fig. 2 lane C vs. lane A), as estimated by both the densitometric tracing method and by counting gel

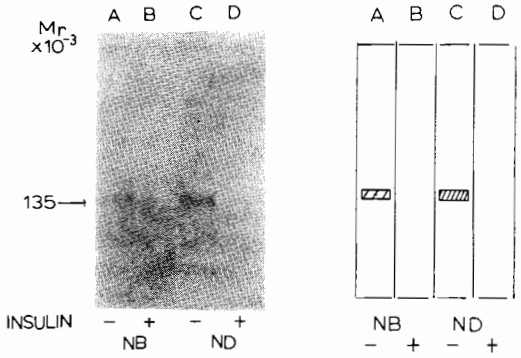

Fig. 2. Autoradiogram of a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoretic pattern of undifferentiated (NB) and differentiated (ND) NB-15 mouse neuroblastoma membranes cross-linked to ^{125}I-labeled insulin. Equal amounts of membranes from undifferentiated (lanes A and B) and differentiated (lanes C and D) were cross-linked with ^{125}I-labeled insulin and electrophoresed in the presence of 15% β-mercaptoethanol. During cross-linking reaction, membrane samples in lanes B and D were incubated with 5 μM unlabeled insulin. Samples of affinity-labeled membranes were electrophoresed in the presence of 10% β-mercaptoethanol. Each lane contained membranes equivalent to 50 μg proteins.

slices excised from the gel. We have also found that excess unlabeled nerve growth factor (1 μM) did not compete with ^{125}I-labeled insulin for the same binding sites on cross-linking reaction (data not shown).

Quantitative comparison of the insulin receptors of the undifferentiated and differentiated NB-15 cells was made by determining specific insulin binding over the concentration range 0.5–1000 nM insulin in intact cells. A typical set of binding data, plotted according to the method of Scatchard [20], is presented in Fig. 3. Both the undifferentiated and differentiated NB-15 cells yield curvilinear plots suggestive of heterogeneity of sites [21] or negative cooperativity [22]. Since the curvature occurred at insulin concentrations greater than 40 nM, only the high-affinity binding is considered to be physiologically relevant [23]. The number of binding sites was calculated from the intercept on the abscissa and the cell number used in the assays, while the equilibrium dissociation constant (K_d) was determined from the slope of the plot [24]. The results thus obtained indicated that for the undifferentiated NB-15 cells there were ap-

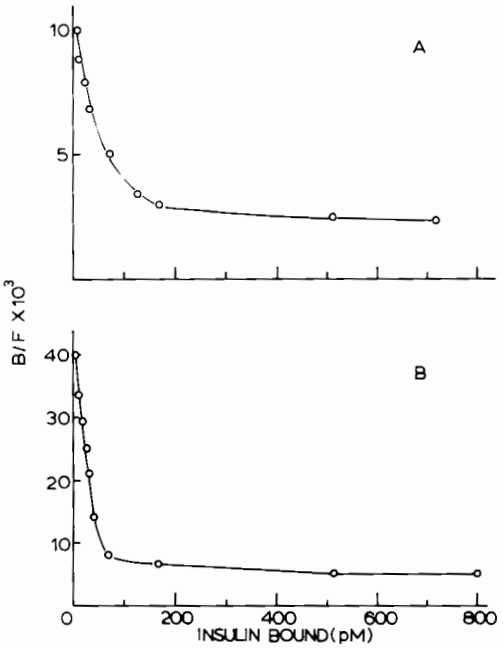

Fig. 3. Scatchard plots of insulin binding data for undifferentiated (A) and differentiated (B) NB-15 mouse neuroblastoma cells. Undifferentiated NB-15 cells ($5.5 \cdot 10^6$ cells/ml) and differentiated NB-15 cells ($5.0 \cdot 10^5$ cells/ml) were incubated to equilibrium at 17°C in a binding assay buffer containing mixtures of ^{125}I-labeled insulin (450 cpm/fmol) and nonradioactive insulin over the concentration range 0.5–1000 nM. Insulin binding activity was determined, and all data were corrected for nonspecific binding.

prox. 7200 high-affinity sites per cell, with a K_d value of 6.5 nM, whereas for the differentiated NB-15 cells, there were approx. 45000 sites per cell and a K_d value of 1.2 nM. The results presented in both Fig. 2 and Fig. 3 suggested that there were more copies of insulin receptor in the differentiated NB-15 cells than in the undifferentiated NB-15 cells. However, despite a 6-fold increase in receptor number per cell and a 5-fold increase in affinity, as determined by binding assay (Fig. 3), there was only a 1.5-fold increase in labeling intensity of the affinity labeled α-subunits of insulin receptor (Fig. 2). This qualitative discrepancy may be due to (a) difference in cell size between undifferentiated and differentiated NB-15 cells [2], such that the number of receptors per unit membrane may not be so different between these two cell types, and (b) difference in the experimental conditions used for affinity labeling and for binding assay.

Lack of response of mouse neuroblastoma cells to the effect of nerve growth factor in inducing neurite outgrowth has been reported [25]. However, some previous studies suggested that nerve growth factor may specifically bind to the cell surface of mouse neuroblastoma cells [26]. In order to ascertain this, the possible existence of nerve growth factor receptor in NB-15 mouse neuroblastoma cells was examined using the technique of affinity labeling. PC-12 rat pheochromocytoma clonal line, a well-known nerve growth factor responsive cell line, was used in this experiment as a control. The results are illustrated in Fig. 4. Using membranes from PC-12 cells and performing binding to ^{125}I-labeled nerve growth factor followed by cross-linking with disuccinimidyl suberate resulted in a specific labeling of two polypeptide bands on sodium dodecyl sulfate-polyacrylamide gel with apparent molecular weights of 145000 and 115000 (Fig. 4, lanes A–C). The M_r 115000 species was more prominently labeled than the M_r 145000 species (Fig. 4, lane B). These data are in good agreement with results obtained by Massague et al.

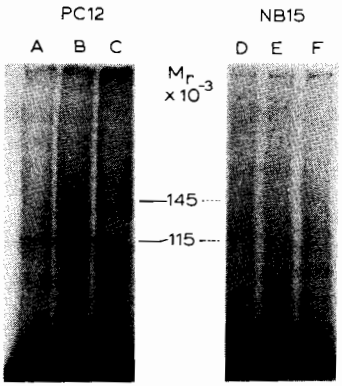

Fig. 4. Autoradiogram of sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns of PC-12 rat pheochromocytoma membranes (lanes A, B and C) and NB-15 mouse neuroblastoma membranes (lanes D, E and F) cross-linked to ^{125}I-labeled nerve growth factor. Membrane samples in all lanes were incubated with 2 nM ^{125}I-labeled nerve growth factor. Samples in lanes A and D were not treated with cross-linking agent disuccinimidyl suberate whereas samples in lanes C and F were incubated with 0.5 μM unlabeled nerve growth factor. Sample in each lane contained membranes equivalent to 50 μg proteins.

[27]. They have recently reported that, in the presence of the photoactive bifunctional agent hydroxysuccinimidyl-*p*-azidobenzoate, ^{125}I-labeled nerve growth factor labels an M_r 148 000–158 000 polypeptide in intact PC-12 cells, but an M_r 120 000–130 000 species in membranes prepared from PC-12 cells. Under identical labeling conditions, no defined bands could be clearly demonstrated in NB-15 cells (Fig. 4 lanes D–F), suggesting that either the number of nerve growth factor receptors in NB-15 cells was far less than that in PC-12 cells or alternatively, nerve growth factor receptor was absent in NB-15 mouse neuroblastoma cells.

Discussion

Insulin has been shown to affect directly the central nervous system (CNS) function by producing hypoglycemia [28]. Specific binding of ^{125}I-labeled insulin to membrane preparations from whole brain of animals or primary neuronal cells has been previously reported [29,30]. Yip et al. [31] have shown that, following electrophoresis of photoaffinity labeled protein from whole brain homogenates, a protein with an apparent M_r of 115 000 is the predominately labeled species. Heidenreich et al. [31], have confirmed this finding and further localized the insulin receptor in various regions of rat brain.

It has been well documented that differentiated mouse neuroblastoma cells can be used as an in vitro model for neuronal cells [2–4,33]. The results described in this paper demonstrated for the first time the presence of insulin receptors on both undifferentiated and differentiated NB-15 mouse neuroblastoma cells (Figs. 1 and 2). The insulin receptor subunit identified in mouse NB-15 neuroblastoma cells has an apparent M_r of 135 000, which is similar to that of the α-subunit of insulin receptor in many other cell types (e.g., Ref. 17), but which is different from that of M_r 115 000 α-subunits in rat brain [31,32]. Direct comparison of insulin receptors of mouse neuroblastoma cells and mouse brain under identical experimental conditions should ascertain whether there are true differences in insulin receptors between mouse neuroblastoma cells and mouse brain cells.

Insulin was found to be an essential component in the chemically defined serum-free medium developed for the growth of rat and mouse neuroblastoma cells [5]. Spoerri et al. [6] have shown that Neuro-2a mouse neuroblastoma cells produce more extensive and elaborate neurite outgrowth in this insulin-containing medium. Related to this finding is a preliminary report that insulin and insulin-like growth factor-II stimulate neurite formation in cultured human neuroblastoma cells [34]. These studies, together with the identification of insulin receptors on mouse neuroblastoma cells (Fig. 1), suggest that insulin receptor may have a physiological role in the survival and development of neuronal cells.

Our studies also indicated an approx. 6-fold increase of insulin binding sites per cell in differentiated NB-15 cells as compared to that in the undifferentiated NB-15 cells (Fig. 3). This finding is interesting in light of the studies by Rubin et al. [35]. They have shown that 3T3-L1 preadipocytes increase the number of receptors per cell from 6500 to 250 000 upon differentiation with a concomitant change of K_d values from 0.8 nM to 4 nM. Parallel physiological studies also show that insulin receptors of differentiated 3T3-L1 cells are more effectively coupled to the hexose transport system and glucose-metabolizing enzyme systems [35]. The increase of insulin binding sites in the differentiated NB-15 cells was not as dramatic as that in the differentiated 3T3-L1 cells (6-fold compared to 40-fold). Nevertheless, this increase of insulin binding sites in differentiated NB-15 cells may provide a biochemical basis for the apparently more effective action of insulin in potentiating the asparagine-mediated ornithine decarboxylase induction in the differentiated NB-15 cells than in the undifferentiated cells (Chen and Rinehart, unpublished data).

Nerve growth factor is known as an important hormone involved in the survival, development and differentiation of various cell types of neural crest origin [36,37]. Our studies, however, indicated that nerve growth factor receptor was demonstratable by chemical affinity labeling technique in PC-12 rat pheochromocytoma cells, but not in NB-15 mouse neuroblastoma cells (Fig. 4, lane B vs. lane E). This finding may be related to the fact that nerve growth factor promotes neurite outgrowth in PC-12 cells, but not in mouse neuroblastoma cells.

Acknowledgments

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