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Unglycosylated Trk protein does not co-localize nor associate with ganglioside GM1 in stable clone of PC12 cells overexpressing Trk (PCtrk cells)

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Our previous studies have shown that acidic glycosphingolipid, ganglioside GM1 (GM1), is an endogenous regulator of high affinity nerve growth factor receptor, Trk, which is an essential factor for the normal development and differentiation of neuronal cells by forming a complex with Trk. GM1 is also known to be a major constituent of caveola or glycosphingolipid-enriched microdomain (GEM) of the plasma membrane. In order to study the effect of the glycosylation of Trk on the formation of GM1-Trk complex and subcellular distribution of this protein, we generated PC12 cells stably overexpressing Trk (PCtrk). Pretreatment of this stable clones with tunicamycin, a potent inhibitor of N-glycosylation, caused the appearance of unglycosylated Trk core protein. These unglycosylated Trk can hardly respond to its ligand, NGF. Sucrose density gradient analysis revealed that unglycosylated Trk core protein was recovered in high density fractions, whereas most of GM1 is present in low density fractions corresponding to caveola or GEM fractions. Moreover, these unglycosylated Trk proteins lose their ability to form a complex with GM1, although GM1 is present in the same high density fractions. These data strongly suggest that spatial segregation of GM1 from the Trk protein by the inhibition of the glycosylation of Trk might be an important molecular mechanism for the unresponsiveness to NGF. Moreover, the binding site of GM1 in the Trk protein might act as an important determinant for the normal trafficking of the Trk protein within the cells.

Keywords: **Trk, ganglioside GM1, signal transduction, glycolipids enriched microdomain (GEM), glycosylation, tunicamycin**

Introduction

Accumulating evidences have suggested that the plasma membrane is not homogenous but contains specific subcompartments characterized by their unique lipid and protein composition [1,2]. Based on their enrichment in various signalling molecules, these microcompartments have been postulated to be sites for localized signal transduction for several external stimuli [3–5]. Caveola contain high amounts of cholesterol, acidic glycosphingolipids such as ganglioside GM1 (GM1), sphingomyelin, and an important structual protein for its formation called as caveolins. Withdrawal of cholesterol by filipin and nystatin has been reported to result in the disappearance of caveola structure [3–5]. The other membrane microdomain called as glycolipid-enriched microdomain (GEM) lacks structural protein caveolin but contains enriched amounts of gangliosides and signaling molecules such as src tyrosine kinase and Ras proteins [6]. Our previous studies and others have demonstrated that high affinity neurotrophic factor receptors such as Trk-family tyrosine kinase receptors and low affinity nerve growth factor receptor, p75NTR are present in these subcompartment of the plasma membrane [7,8]. At present, however, the real roles of these special membrane compartments on Trk-dependent intracellular signal transduction pathway remain to be elucidated.

The neurotrophins influence survival and maintenance of vertebrate neurons in the embryonic, early post-natal and post-developmental stages of the nervous system [9]. High affinity nerve growth factor receptor (NGF), Trk, has an intrinsic tyrosine kinase activity which is located in the intracellular domain. The initial step of the intracellular signal transduction of NGF is believed to be the activation of Trk-associated tyrosine kinase activity by the autophosphorylation on tyrosine residues [10,11]. Our previous studies have shown that this important membrane subcompartment constituent, GM1

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enhances the action of NGF by enhancing NGF-induced autophosphorylation of Trk [12,13]. These enhancing effect of GM1 is at least in part due to a tight association of GM1 with the Trk protein in a way that is SDS-resistant [13], although the precise molecular mechanism of this interaction and the binding site(s) of GM1 in the Trk protein remained to be unknown. Subsequently, we found that the Trk receptor is no more responsive to its ligand in chemically GM1-depleted PC12 cells by glucosyl ceramide synthase inhibitor (D-PDMP) [14]. These previous findings strongly suggest that Trk needs an appropriate lipid cofactor such as GM1 which is located near Trk in the plasma membrane for its normal function. Trk contains thirteen potential glycosylation sites in its extracellular domain and is in fact highly glycosylated by the normal post-translational modification. Recent work by Watson et al. has suggested that glycosylation of the Trk protein regulates receptor localization. Unglycosylated Trk protein is trapped intracellularly and NGF is unable to induce tyrosine autophosphorylation of Trk-associated tyrosine kinase nor to activate kinases in the Ras-MAPK pathway [15].

In this study, we examined the effect of the N-glycosylation of the Trk protein on the association of GM1 with Trk and the receptor activity in PC12 cells. The data clearly suggested that GM1 is not associated with unglycosylated Trk core protein which was present in high density fractions and that tunicamycin treatment resulted in the unresponsiveness to NGF in PCtrk cells. These spatial separation of GM1 and Trk might be a molecular basis of the intracellular trapping of unglycosylated Trk and its unresponsiveness to NGF.

Materials and methods

Cell culture and transfection of human *trk* cDNA

Rat pheochromocytoma cell-line, PC12 cells were cultured as monolayers in culture dishes with Dullbecco's modified Eagle's medium (DMEM) containing 7.5% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin as described previously [12–14]. pRcCMV-*trk* plasmid containing human *trk* cDNA was kindly provided by Dr. Guroff G (NICHD, National Institutes of Health, Bethesda, MD, USA) [16]. The expression vector containing human *trk* cDNA or vector alone (mock) were transfected into PC12 cells using lipid liposome, Lipofectamine (Gibco-BRL), and stable clones were selected under selection medium containing 300 μg/ml G418 (Life Technologies, Inc. USA). Stable clones expressing Trk (PCtrk) were screened by Western blot analysis as described previously [12–14] and maintained in the regular growth medium containing 300 μg/ml G418.

Sucrose density gradient analysis

Sucrose density gradient analysis was performed essentially according to the method described [19]. PCtrk cells were

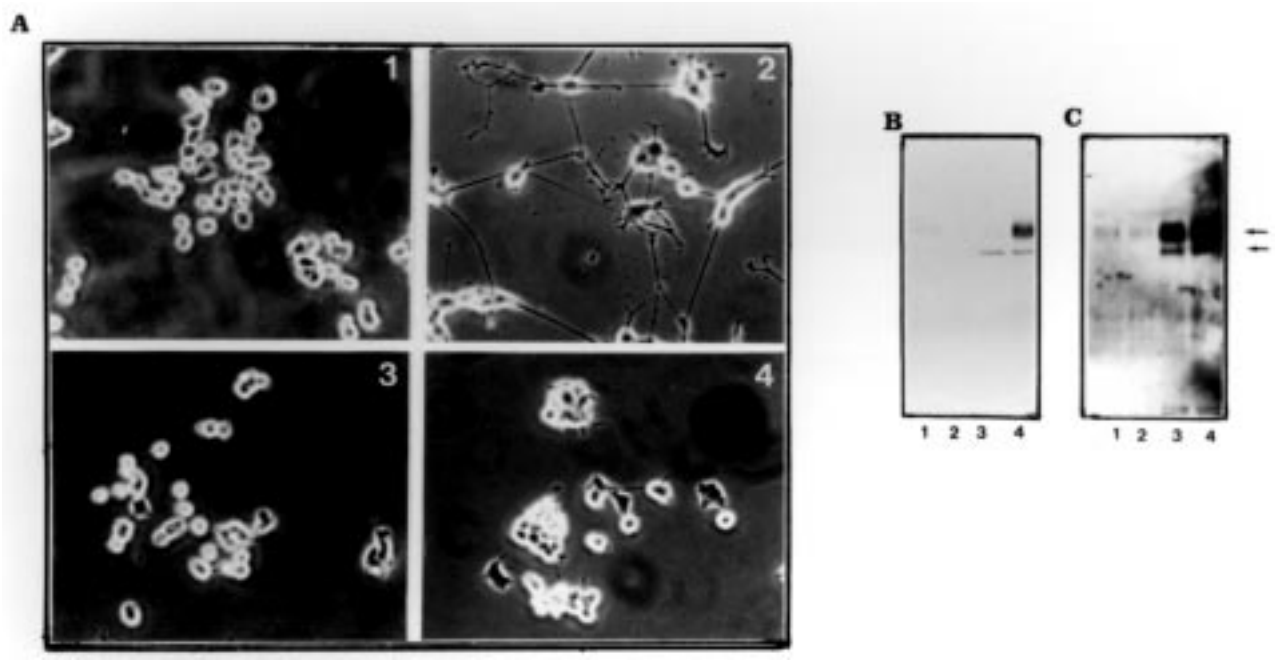

Figure 1. Enhanced morphological differentiation (A) and autophosphorylation by NGF in PC12 cells stably overexpressing Trk (PCtrk). **A**. PCtrk cells [1,2] and mock-transfected PC12 cells [3,4] were cultured on 25:1 collagen/poly-L-lysine-coated 24-well plates in the regular medium. The cells were stimulated with 50 ng/ml NGF [2,4] for 1 day. The pictures are shown as phase contrast micrographs made from typical areas of these cultures. **B**. PCtrk cells (lanes 3, 4) and mock-transfected PC12 cells (lanes 1, 2) were cultred on 175 cm^2 flasks in the regular medium and were serum-starved for 1 h before the stimulation with NGF 50 ng/ml for 5 min (lanes 1, 4). Trk was immunoprecipitated with an anti-Trk antibody, and Western blot analyses were performed on these Trk-immunoprecipitates with an anti-phosphotyrosine antibody. **C**. PVDF membranes used in B were reprobed with an anti-Trk antibody after stripping the first antibody as specified in a manual from the manufacturer.

placed on ice briefly and then GM1 in the cells was labelled with 10 μg/ml horse-raddish peroxidase (HRP)-conjugated B subunit of cholera toxin (CTB) for 15 min at 4°C. Then the cells were homogenized using a Teflon glass homogenizer in TNE/Triton X-100 buffer (1% Triton X-100, 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA). The lysate was brought to 1.5 M sucrose. Discontinuous sucrose gradient (1.2 M, 8.5 ml; 0.15 M, 2.5 ml) in TNE buffer without Triton X-100 was layered over the lysate. Gradients were centrifuged for 18 h at 38 000 rpm at 4°C in a Hitachi ultracentrifuge. 1-ml fractions and the pellet were collected and used for HRP activity determination or Western blot analysis.

Immunoprecipitation and immunoblot analysis

Immunoprecipitation was performed essentially as described previously [13,14]. In brief, control and NGF-treated PCtrk cells were lysed in lysis buffer (20 mM Hepes, pH 7.2, 1% Nonidet P-40, 10% (v/v) glycerol, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 10 μg/ml leupeptin). Cell-free lysates were normalized for proteins (usually 1 mg/ml) and Trk was immunoprecipitated with an anti-Trk antibody from cell-free lysates as described above [14]. The resulting immunoprecipitates were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes after SDS/polyacrylamide gel electrophoresis on 10% gels and were probed with an anti-Trk antibody or anti-phosphotyrosine antibody (4G10, Upstate Biotechnology Inc. USA). Detection of positive bands was performed using Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products, Inc. USA).

Lipid extraction from Trk-immunoprecipitates

Total lipids are extracted from Trk-immunoprecipitates by use of choloform : methanol (2:1) and further fractionated by use

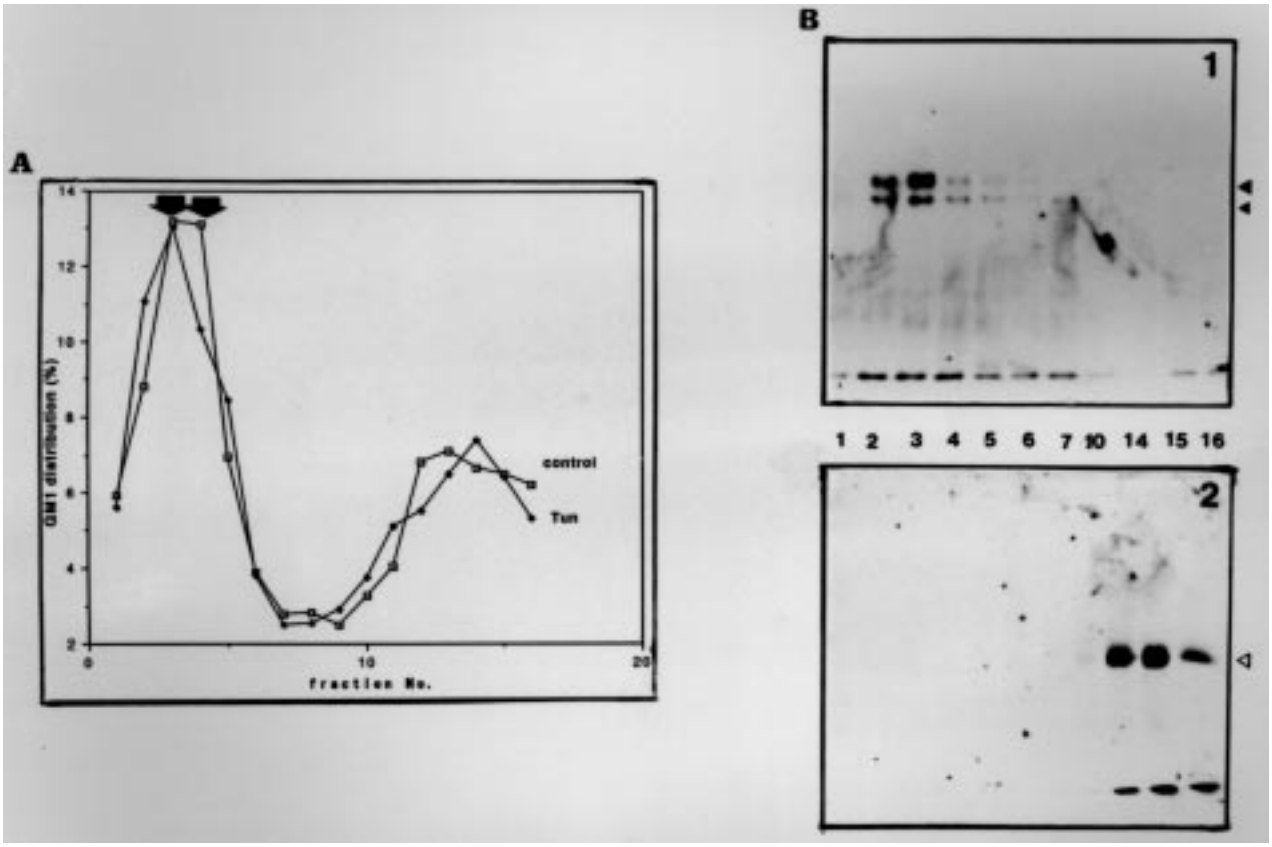

Figure 2. Sucrose density gradient ultracentrifugation of PCtrk cells cultured in the presence or absence of 5 μg/ml tunicamycin for 12 h at 37°C and then labelled with 10 μg/ml HRP-conjugated CTB for 15 min at 4°C. **A**. PCtrk cells were cultured on 175 cm^2 flasks in the presence (tun) or absence (control) of 5 μg/ml tunicamycin for 12 h at 37°C. Then, the cells were labelled with HRP-conjugated CTB for 15 min at 4°C. The cells were processed as described in 'Materials and methods' and same amount of protein (1 mg) from each cell lysates obtained by TNE/Triton X-100 lysis buffer were subjected to sucrose density gradient ultracentrifugation. 1-ml fractions and pellet were collected and subjected to HRP activity determination. **B**. Each fraction obtained from A were subjected to Western blot analysis probed with an anti-Trk antibody. 1, samples from PCtrk cells cultured in the absence of tunicamycin; 2, samples from PCtrk cells cultured in the presence of tunicamycin for 12 h at 37°C. Closed triangles indicate the position of 140 kDa mature Trk and 110 kDa immature Trk protein. Open triangle indicates the position of 75 kDa unglycosylated Trk core protein. The numbers between figures indicate the fraction numbers of sucrose density gradient analysis.

of Sep-pak C18 culumn as reported previously. The obtained samples were subjected to ascending thin-layer chromatography developed in chloroform/methanol/0.02% $CaCl_2$ (55/45/10, v/v/v) by using TLC plate (Polygram siL, N-HR, Macherey-Nagel, Germany). Idenfication of GM1 on the TLC plate was performed as reported previously [13].

Tunicamycin treatment

PCtrk cells were pretreated with tunicamycin (5 μg/ml) for 12 h at 37°C. Then, cells were treated as described above and were lysed with lysis buffer. Immunoprecipitation with an anti-Trk antibody and Western blot analysis was conducted as described above.

Results and discussion

In order to elucidate the effect of glycosylation of the Trk protein on its association with GM1 and the receptor function, we generated a stable transfectant of *trk* cDNA in PC12 cells. Heretofore, these PC12 cells overexpressing Trk (PCtrk) have been successfully used in many studies to examine the effect of NGF and Trk-initiated intracelluar signalling pathway [17,18]. Stable transfectant of *trk* cDNA (PCtrk) showed more rapid morphological differentiation in response to NGF than that of mock-transfected PC12 cells as reported previously (Figure 1A) [17]. Western blot analysis of Trk from PCtrk and mock-transfected PC12 cells gave the result that PCtrk cells expressed ten-times higher amount of the Trk protein than mock-transfected cells (Figure 1B). Confocal lazor microscopy revealed these overexpressed Trk protein in subcompartment of plasma membrane with pathy staining pattern, where GM1 was also co-localized (data not shown).

As described earlier, GM1 is now known to be enriched in caveola or GEM structure in the plasma membrane, where normal Trk protein is also targetted after post-translational modification. These spacial co-localization of GM1 and Trk in the subcompartment of the plasma membrane might be essential for the normal function of the Trk protein. To test this hypothesis, PCtrk cells were pretreated with tunicamycin, a potent inhibitor of protein N-glycosylation, for 12 h at 37°C and were subjected to sucrose density gradient ultracentrifugation. Tunicamycin-treatment caused total disappearance of mature fully glycosylated Trk protein from the low density fractions (Figure 2B-2). Unglycosylated Trk core protein was found in high density fractions (Figure 2B-2), although 140 kDa mature Trk was present in low density fractions. On the other hand, considerable amount of GM1 was still recovered in low density fractions of samples from cells treated with tunicamycin, although small amount of GM1 was

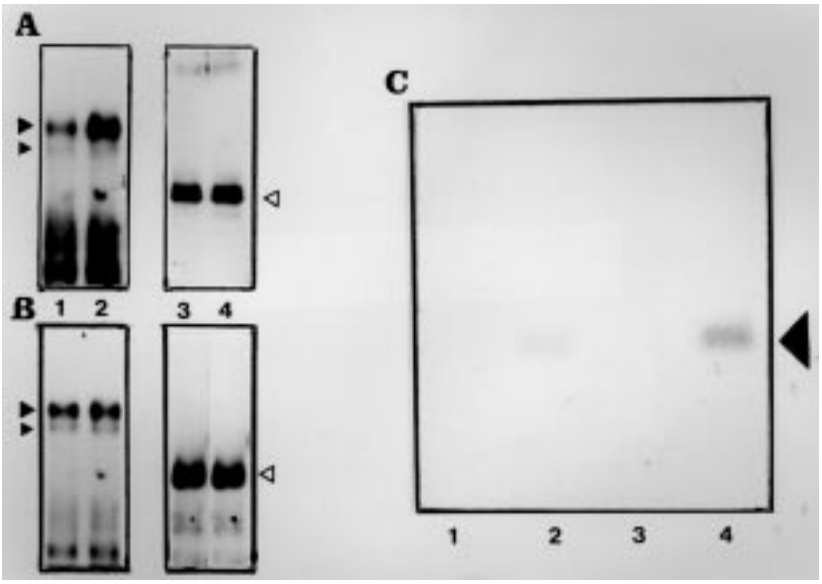

Figure 3. Loss of autophosphorylation on tyrosine residue(s) in response to NGF (A, B) and loss of association with GM1(C) in unglycosylated Trk core proein. **A**. PCtrk cells were preincubated with [3,4] or without [1,2] 5 μg/ml tunicamycin for 12 h. Then cells were cultured in serum-free medium for 1 h with continuous presence of tunicamycin and stimulated with 50 ng/ml NGF for 5 min (lanes 2,4) or left untreated (lanes 1, 3). Trk was immunoprecipitated with an anti-Trk antibody. Western blot analysis was performed on the Trk-immunoprecipitates with an anti-phosphotyrosine antibody and ECL detection system. **B**. The same samples used in A were immunoblotted with an anti-Trk antibody to check the amount of the Trk protein in Trk-immunoprecipitates. Closed tirangles indicate the positions corresponding to 140 kDa mature and 110 kDa immature Trk protein. Open triangle indicates the position of unglycosylated Trk core protein. **C**. Total lipids were extracted from Trk-immunoprecipitates obtained from cells pretreated with 50 μg/ml tunicamycin [1,3] or untreated [2,4] for 12 h and then stimulated with 50 ng/ml NGF as described under 'Materials and methods'. Trk-immunopecipitates were obtained with an anti-Trk antibody from cell-free lysates from the above-mentioned cells. Lipid extraction from these Trk-immunoprecipitates was performed as described under 'Materials and methods'. Closed triangle indicates the position corresponding to GM1.

present in high density fractions (Figure 2A). At present, the identity of GM1 in high density fractions is unknown.

We next examined the tyrosine phosphorylation of Trk induced by NGF. We hardly observed tyrosine phosphorylation response to NGF on unglycosylated Trk core protein (Figure 3A). We also extracted total lipids from Trk-immunoprecipitates from tunicamycin-treated and untreated PCtrk cells. To our surprise, we could not detect any GM1 in samples from tunicamycin-treated cells, although we clearly observed GM1 in samples from untreated cells (Figure 3C-2, 4). These data strongly suggest that unglycosylated Trk core protein in high density fractions of sucrose density gradient does not have any interaction with GM1 present in these high density fractions. These findings well contrast with our previous data that GM1 can form a complex with normal fully glycosylated mature Trk protein and regulate it positively [13]. At this point, recent report by Watson et al. is extremely interesting [15]. They found that glycosylation of the Trk protein is necessary for its normal targeting to the plasma membrane and unglycosylated Trk core protein is no more responsive to NGF. The molecular mechanism of the unresponsiveness to its ligand, NGF, however, remained to be elucidated. The present findings strongly suggest that these spatial segregation of the Trk protein from GM1 and loss of GM1 binding activity of unglycosylated Trk core protein might be, at least in part, an important molecular mechanism for the unresponsiveness to NGF. Moreover, these results might open an intriguing possibility that other growth factor receptors might be regulated in a same way. Tunicamycin is also known to be an inducer of so called unfolded protein response (UPR) of the endoplasmic reticulum [20]. Therefore, in case of UPR, the same trouble might occur in the maturation step of the Trk protein. We are currently investigating these possibilities.

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