Sphingolipids


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Gangliosides and growth factor receptor regulation
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Introduction
Gangliosides are sialic acid-containing glycosphingolipids that are found largely in the outer leaflet of plasma membranes of virtually all mammalian cells. Although their cellular functions are not clear, gangliosides can alter the physicochemical properties of cell membranes, such as membrane fluidity [2] and electrostatic surface potential [3], and affect major cellular processes including proliferation, differentiation, survival and apoptosis [4–6]. Changes in ganglioside composition are also correlated with development, aging and neoplastic transformation [5–7].

Many of the biological effects of gangliosides are caused by their interactions with membrane-bound proteins, usually through the oligosaccharide portion of gangliosides. Because over

Abbreviations used: (EGF)R, epidermal growth factor (receptor); NGF, nerve growth factor; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor. Ganglioside nomenclature is that of Svenneholm [1].

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100 distinct gangliosides differing in their oligosaccharide chains have been identified in vertebrate cells, a variety of different proteins might be able to interact with specific gangliosides. In many cases these proteins are also involved with cellular signal transduction, examples of which include receptor tyrosine kinases, opioid receptors, β1-adrenergic receptors, Srcs and integrins [6,8,9]. We and others have discussed the details of how these proteins are modulated by gangliosides [6,8,9]; here we highlight recent progress made on the regulation by gangliosides of the platelet-derived growth factor receptor (PDGFR) and the high-affinity nerve growth factor (NGF) receptor, TrkA.

**Regulation of PDGFRαβ**

Several sets of experiments implicate gangliosides in the modulation of cell growth. These include those showing changes in ganglioside composition as cultured cells approached confluence and those showing that exogenously added gangliosides decreased cell proliferation and saturation density, and increased cell cycle length [6,10,11]. This led to the demonstration that exogenously added gangliosides could inhibit growth-factor-mediated cellular proliferation [12]. Further biochemical studies identified several receptor tyrosine kinases, such as PDGFR and epidermal growth factor receptor (EGFR), as potential inhibitory targets of ganglioside action [6].

Platelet-derived growth factor (PDGF) is a potent mitogenic agent and consists of homologous A and B chains that associate as a heterodimer (AB) and as homodimers (AA, BB) [13]. Two closely related receptors, PDGFRα and PDGFRβ, bind PDGF dimers with different affinities and can themselves associate as homodimers (αα, ββ) or heterodimers (αβ) [13,14]. Monomeric PDGFR/β binds only the PDGF B chain with high affinity, whereas monomeric PDGFRα binds both PDGF A and B chains. Consequently, PDGF-AB activates all PDGFRs, PDGF-AA activates only αα homodimers, and PDGF-AB stimulates PDGFR and αβ dimers. Exposure of cells to PDGF assembles the monomeric receptor subunits into dimers and triggers specific biochemical events and intracellular signalling cascades [13,14].

Bremer found that certain gangliosides added exogenously to the growth medium inhibit both PDGF- and epidermal growth factor (EGF)-mediated growth of Swiss 3T3 cells [12]. GM3 inhibits both PDGF- and EGF-stimulated mitogenesis, but ganglioside GM1 inhibits only PDGF-stimulated mitogenesis [12]. Ligand binding studies with 125I-PDGFR indicate that the effects of GM1 and GM3 are not due to a decrease in PDGF binding affinity of PDGFR, although they do cause a small increase in the Kd [12]. Neither ganglioside changes the absolute number of receptors on the cell surface or competes directly with PDGF for receptor binding. GM1 inhibited the PDGF-stimulated tyrosine phosphorylation of a 170 kDa protein [12], which was subsequently found by Western blot analysis to be the PDGFR protein [15]. In a chemical cross-linking study, the same authors demonstrated that GM1 inhibited PDGFR dimerization [15]. Inhibition of PDGFR tyrosine phosphorylation and dimerization was further studied with a panel of gangliosides; it was found that although GM3 was ineffective, GM1, GM2, GD1a, GD3 and GT1b were all potent inhibitors of the tyrosine phosphorylation and dimerization of PDGFR. Thus it seems that inhibition of the ligand-induced dimerization of PDGFR is the critical ganglioside-mediated event responsible for the inhibition of its tyrosine kinase activation and several post-receptor biochemical and biological consequences of PDGFR activation.

**GM1 inhibits downstream signalling by PDGFRαβ**

Because many downstream signalling pathways originate directly from tyrosine-phosphorylated PDGFR, it is reasonable to postulate that intracellular signalling cascades are also affected by GM1. This has turned out to be true for most events studied so far. For example, the pretreatment of human glioma and Swiss 3T3 cells with GM1 inhibits PDGF-mediated changes in intracellular Ca2+ concentrations [16] and membrane translocation of protein kinase C [17]. In vascular smooth-muscle cells, GM2 and GM1 inhibited the PDGF-dependent stimulation of phospholipase Cγ, accumulation of inositol 1,4,5-triphosphate, and expression of the immediate-early response gene c-fos [18]. Our recent findings that ligand-induced associations of several proteins with PDGFR are all inhibited by GM1 are consistent with these results (T. Farooqui, A.J. Yates and A.A. Rampersaud, unpublished work). These include interactions between PDGFR and...
the p85 subunit of phosphoinositol 3-kinase, between PDGFR and phospholipase Cγ, and between PDGFR and GTPase-activating protein. We interpret these findings as being due to GM1’s inhibiting the phosphorylation of critical tyrosine residues essential for the docking of signalling proteins to PDGFR. However, we have also found that PDGF-mediated activation of the mitogen-activated protein kinase isoform Erk2 is not affected by pretreatment of cells with GM1 (J. Oblinger, A.A. Rampersaud and A.J. Yates, unpublished work). It is possible that in GM1-treated cells PDGF-stimulated signalling still occurs at a low level and is sufficient to activate mitogen-activated protein kinase fully.

GM1 inhibits the tyrosine phosphorylation of both PDGFRα and PDGFRβ

Although the effects of GM1 on the regulation of PDGFRβ signalling have been studied extensively [19], little is known about the effects of gangliosides on PDGFRα signalling. Both receptors produce similar, although not identical, cellular

**Figure 1**

Inhibition of PDGF-stimulated tyrosine phosphorylation by ganglioside GM1

Serum-starved Swiss 3T3 cells were preincubated for 2 h with the following concentrations of GM1: lanes 1 and 3, 0 μM; lane 4, 10 μM; lane 5, 25 μM; lane 6, 50 μM; lanes 2 and 7, 100 μM. They were then treated for 10 min with (lanes 3–7) or without (lanes 1 and 2) 50 ng/ml PDGF-AA. Cells were lysed in kinase lysis buffer [43] and the resulting postnuclear supernatants were immunoprecipitated (IP) overnight with polyclonal anti-PDGFRα antibodies followed by treatment with Protein A–agarose beads. The immunoprecipitates were resolved by SDS/PAGE and transferred to PVDF membranes; the Western blot (WB) was probed with monoclonal anti-phosphotyrosine antibodies (A). Immunoreactive material was detected with an Amersham chemiluminescence kit. The blot was then stripped and reprobed with polyclonal anti-PDGFRα antibodies (B). Shown is the portion of the blot that resolved proteins in the molecular mass range 80–180 kDa. Lane 8 shows results for cells that were taken through the same procedure except that they were treated with 300 μM sodium pervanadate as a positive control.

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IP: anti-PDGFRα

WB: anti-phosphotyrosine

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IP: anti-PDGFRα

WB: anti-PDGFRα

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responses and probably use the same dimerization mechanism for their activation [14]. However, there are differences in the sets of signalling proteins that associate with each receptor [14], as well as differences in the individual roles of these signalling proteins in cell proliferation [20,21]. Additionally, there are substantial differences in the amino acid sequences of the receptors, particularly in their extracellular region, where there is only 30% sequence similarity [13].

As an initial step in determining whether there is a difference in the way that gangliosides modulate PDGFRβ compared with that of PDGFRα, we studied the effects of GM1 on PDGFR-AA-stimulated tyrosine phosphorylation of PDGFRα. Swiss 3T3 cells express high levels of PDGFRα protein and have been well characterized for PDGFRα function. We incubated Swiss 3T3 cells with increasing concentrations of GM1 for 2 h and then stimulated them with PDGF-AA for 10 min. By using anti-PDGFRα antibodies, immunoprecipitates were prepared from cell lysates; tyrosine phosphorylation of immunoprecipitated proteins was studied by Western blot analysis. Results from a representative experiment are shown in Figure 1 and demonstrate that GM1 significantly inhibits the PDGFR-AA-stimulated tyrosine phosphorylation of PDGFRα. Preincubating cells with 50–100 µM GM1 for 2 h (Figure 1, lanes 6 and 7) resulted in the maximal inhibition of PDGFRα phosphorylation, conditions that are close to those needed for a similar degree of inhibition of PDGFRβ dimerization [15]. This suggests that GM1 inhibits PDGFRα and PDGFRβ through a similar mechanism and that this is likely to involve the inhibition of receptor dimerization.

Role of GM1 in TrkA signalling

A similar body of studies implicates gangliosides in the modulation of growth and differentiation of neural tissues [22]. These include analytical studies in which changes in glycolipids, including ganglioside patterns, were observed during postnatal development in human, mouse and rat brain. On the basis of the results from these studies it was concluded that some gangliosides could be considered as markers of specific cell types and stages of cell maturation [22]. Other studies showed that the exogenous addition of gangliosides to cultures of neuroblastoma, phaeochromocytoma, dorsal root ganglion and fetal chick brain cells promoted neuritogenesis [5,6]. In most experimental models, neuritogenesis is dependent on the presence of NGF, leading to the suggestion that gangliosides exerted their neuritogenic effects by modulating the signal transduction properties of the high-affinity NGF receptor, TrkA. Unlike PDGFR, GM1 was proposed to have a positive effect on TrkA and subsequent signalling events [23–25].

NGF is part of the neurotrophin family of polypeptides, which has important roles in the development and maintenance of the vertebrate nervous system [26–28]. NGF is required for the survival of cultured neurons as well as the development of several neuronal systems, including sympathetic and sensory neurons in the peripheral nervous system and cholinergic neurons in the basal forebrain [26–28]. When applied to rat phaeochromocytoma (PC12) cells, NGF promotes their differentiation into cells phenotypically similar to sympathetic neurons [29]. NGF binds to TrkA with high affinity, thus inducing the dimerization of TrkA monomers and the activation of tyrosine kinase in the cytoplasmic domain of TrkA. Subsequently, TrkA undergoes tyrosine phosphorylation on at least five different tyrosine residues within three domains of the intracellular part of the receptor [26–28]. Tyrosine-phosphorylated residues within the TrkA cytoplasmic domain recruit several signalling proteins to the receptor, such as phosphoinositol 3-kinase, Shc and phospholipase Cγ, which subsequently stimulate signal transduction cascades leading to neuritogenesis [26–28].

Exogenously added GM1 enhances NGF-stimulated neuritogenesis in PC12 cells without affecting the binding of NGF to TrkA [23,30]. However, GM1 alone does not stimulate neuritogenesis [30]. GM1 also prevents apoptosis in PC12 cells deprived of serum, and promotes the long-term survival of rat sympathetic neurons deprived of NGF [5,31,32]. In comparison with NGF alone, GM1 by itself causes the tyrosine phosphorylation of TrkA over a longer duration and to a smaller degree than does NGF [25]. Taken together, these studies suggest that GM1 might be an allosteric effector of interactions between NGF and TrkA.

Detailed studies have demonstrated that GM1 stimulates a severalfold increase in NGF-stimulated TrkA phosphorylation compared with NGF alone [23–25]. Subsequent cross-linking studies with bis-suberimidate showed that GM1 enhanced the NGF-mediated dimerization of TrkA [23]. In PC12 cells, inhibition of ganglioside
biosynthesis prevented NGF-induced neurite outgrowth and NGF-induced autophosphorylation of Trk [33]. Both of these processes were restored by the addition of GM1 to the culture medium [33]. Although these studies demonstrate that GM1 potentiates the effects of NGF by enhancing the dimerization and activation of the TrkA receptor, not all of the effects of gangliosides seem to be directly attributable to TrkA. In mutant PC12 cells that lack TrkA receptors, TrkB also seems to facilitate cell survival by GM1; however, the mechanism for this remains unknown [31].

**Novel chimaeric proteins that are allosterically regulated by gangliosides**

Experimental evidence shows that gangliosides regulate the signalling activities of PDGFR by inhibiting the dimerization of receptor monomers, and of TrkA by facilitating receptor dimerization. This indicates that allosteric effects of gangliosides on dimerizable receptors are determined by the nature of the receptor itself. Studies in a variety of cell lines have consistently shown that GM1 inhibits the activation of PDGFR regardless of cell type [15,34] or animal species [15,18] or whether the final biological outcome of PDGFR signalling is neuritogenesis or proliferation [34,35]. Such results are most easily explained by assuming that there is a functional domain within PDGFR for interaction with gangliosides.

By using a domain-exchange strategy we are working to identify domains on PDGFRβ and TrkA that interact with gangliosides. Both receptors have the same topology and organization within the membrane, and both require dimerization and tyrosine phosphorylation for receptor activation. We have created chimaeric proteins that have different combinations of extracellular,
transmembrane and cytoplasmic domains from TrkA and PDGFRβ. It is assumed that each chimaera will have biochemical and biological properties that are characteristic of the same domain in the native receptor, including regulation by gangliosides. Thus, depending on the chimaeric receptor, the specific ganglioside added to the culture medium and the nature of the interaction between ganglioside and receptor, novel biological responses can be generated in cells expressing these chimaeric receptors. Results of studies in which these chimaeric receptors are exposed to different combinations of gangliosides and ligands should provide insight into the regulation of PDGFR and TrkA signalling by gangliosides.

One chimaera that we have created is designated Trk-Trk-β (Figure 2) and consists of the extracellular and transmembrane domains (amino acid residues 1-433) from TrkA fused with the last 553 residues of the PDGFRβ cytoplasmic domain (residues 554-1106). Using PC12-nnr5 cells,

**Figure 3**

Neurite formation in PC12-nnr5 cells expressing TrkA and Trk-Trk-β receptors

PC12-nnr5 cells were transfected with retrovirus encoding TrkA and Trk-Trk-β genes. Cells were selected for G418 resistance, then subjected to neuritogenesis experiments on 35 mm collagen-coated dishes. Subconfluent cells expressing TrkA (A, C, E) and Trk-Trk-β (B, D, F) were left untreated (A, B), treated with 100 ng/ml NGF (C, D) or preincubated with 100 μM GM1 before treatment with NGF (E, F). Cells were inspected daily for neuritogenesis. Cultures were photographed after 2 days of treatment, which is when there were the most cells bearing neurites.
which do not make TrkA and do not respond to NGF [36], we created stable cell lines that express Trk-Trk-β and also others expressing wild-type TrkA. The recombinant Trk-Trk-β protein migrates as an approx. 150 kDa protein (between that of 140 kDa TrkA and 180 kDa PDGFR) and undergoes tyrosine phosphorylation in response to NGF (results not shown). Thus NGF’s binding to the extracellular TrkA domain transmits a signal through the TrkA transmembrane region to activate the PDGFRβ tyrosine protein kinase in the cytoplasmic region.

Figure 3 shows preliminary results from studies on neuritogenesis comparing transfected PC12-nnr5 cells expressing Trk-Trk-β with transfected PC12-nnr5 cells that express TrkA. Neither cell line produces neurites in the absence of NGF. However, there is considerable neurite formation by both transfected cell lines in response to NGF added to the culture (Figures 3C and 3D). Most importantly, both cell lines treated with both GM1 and NGF (Figures 3E and 3F) look qualitatively similar to cells treated with NGF, suggesting that GM1 does not inhibit the activation of Trk-Trk-β, although the signalling domain is from PDGFRβ. These results are consistent with the idea that the effects of GM1 on the dimerization and activation of these tyrosine kinase receptors is being exerted through the extracellular and/or transmembrane domains.

Conclusions

We have summarized results that suggest that gangliosides allosterically regulate the signalling properties of two receptor tyrosine kinases, PDGFR and TrkA. This raises the question about whether there are situations in vivo in which gangliosides and receptor tyrosine kinases are in close enough proximity for direct interactions. It is interesting to note that in some cells gangliosides, particularly GM1, are highly enriched in flask-shaped invaginations of the plasma membrane known as caveolae [37,38], which are also sites of multiple signal-transducing molecules, including PDGFR and EGFR [39]. Because caveolae are relatively small (70–100 nm) [37], clustering of dimerizable receptors in these structures increases the possibility of non-ligand-mediated dimerization and receptor activation. Thus gangliosides might provide a means of preventing or facilitating spontaneous dimerization between proximal receptors and could have an important role in regulating receptor activation in the absence of trophic factors. Consistent with this is the recent finding of Bilderback et al. [40] that caveolin, a major protein in caveolae, inhibits TrkA signalling. It is possible that GM1 counteracts the negative effects of caveolin on TrkA.

There is considerable evidence that gangliosides might have important biological roles in modulating cell signalling. Transgenic mice that lack a key enzyme (GM2/GD2 synthase) needed for the biosynthesis of complex gangliosides were initially reported to have only minor behavioural and neurophysiological abnormalities [41]. However, more recent studies have shown that these transgenic mice are unable to transport testosterone to the seminiferous tubules and bloodstream from Leydig cells, resulting in male sterility [42]. Thus gangliosides have a critical role in regulating spermatogenesis; it is likely that further research will identify other essential biological processes that require these interesting but complex molecules for normal function.

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Membrane flow, lipid sorting and cell polarity in HepG2 cells: role of a subapical compartment

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Introduction

The diversity of lipids in eukaryotic cells is in all likelihood a reflection of their diverse functions, of which several are known but many remain to be resolved. Such functions include their ability to modulate protein functioning (even at the level of transcription), their role in signal transduction and their ability to regulate intracellular transport. Inherent to these diverse functions are their specific locations, although in this respect, too, issues such as their asymmetric transbilayer distribution in (intra-)cellular membranes, compositional differences in distinct organelar membranes, and/or their lateral distribution in such membranes, are still largely obscure. Moreover, in the context of the dynamics of (intra-)cellular membranes, it is imperative that mechanisms are

Abbreviations used: ARE, apical recycling endosome; BC, bile canicular membrane; Cer, ceramide; GlcCer, glucosylceramide; MDCK, Madin–Darby canine kidney; NBD, 7-nitrobenz-2-oxa-1,3-diazole; SM, sphingomyelin; SAC, subapical compartment.

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