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Regulation of Integrin Growth Factor Interactions in Oligodendrocytes by Lipid Raft Microdomains

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Summary

Individual growth factors can regulate multiple aspects of behavior within a single cell during differentiation, with each signaling pathway controlled independently and also responsive to other receptors such as cell surface integrins. The mechanisms by which this is achieved remain poorly understood. Here we use myelin-forming oligodendrocytes and their precursors to examine the role of lipid rafts, cholesterol and sphingolipid-rich microdomains of the cell membrane implicated in cell signaling [1]. In these cells, the growth factor PDGF has sequential and independent roles in proliferation and survival [2, 3]. We show that the oligodendrocyte PDGFα receptor becomes sequestered in a raft compartment at the developmental stage when PDGF ceases to promote proliferation, but is now required for survival. We also show that laminin-2, which is expressed on axons in the CNS and which provides a target-dependent signal for oligodendrocyte survival by amplification of PDGFαR signaling [4], induces clustering of the laminin binding integrin α6β1 with the PDGFαR-containing lipid raft domains. This extracellular matrix-induced colocalization of integrin and growth factor receptor generates a signaling environment within the raft for survival-promoting PI3K/Akt activity. These results demonstrate novel signaling roles for lipid rafts that ensure the separation and amplification of growth factor signaling pathways during development.

Results and Discussion

Oligodendrocyte differentiation is associated with expression of at least two distinct sets of lipid rafts [5, 6] and the presence of signaling molecules such as the src family kinase fyn within the raft compartment [7]. In light of this, we hypothesized that these rafts might play a role in defining the downstream signaling response to PDGF. We therefore first asked to what extent the PDGFαR was associated with lipid rafts at different stages of differentiation. We isolated oligodendroglial rafts from Triton X-100 extracts prepared at 4°C based on their property to float as DIGs (detergent-insoluble glycosphingolipid-rich complexes) during density centrifugation [5, 6]. We analyzed four stages of differentiation (Figure 1A)—early progenitors (O2A cells), late progenitors (characterized by expression of the O4 marker), newly formed oligodendrocytes (expressing galactocerebroside [GalC+]), and mature oligodendrocytes (expressing myelin basic protein [MBP+]). Western immunoblot analysis revealed the presence of the PDGFαR within both fractions at all stages, although only very low levels were seen at the latest (MBP) stage of differentiation (Figure 1B). There was a significant shift in the balance of the two fractions at the stage of initial differentiation, with a 3-fold increase in the relative level within the DIG fraction of newly formed GalC+ oligodendrocytes (Figure 1C). This shift in receptor distribution at the stage of GalC expression was independent of cell culture substrate (data not shown, but see Figure 3A). GalC expression defines the end of oligodendrocyte precursor proliferation and the start of terminal differentiation, at which developmental stage many of the cells will undergo apoptosis [3, 8]. We conclude that the switch in PDGF function from mitogen to survival factor correlates with a sequestration of the receptor in a raft fraction.

As α6β1 integrin enhances PDGF survival signaling, so providing a mechanism by which axonal cues regulate oligodendrocyte survival [4], we next examined if α6β1 is also a raft-associated protein in the newly formed oligodendrocytes. Western blotting experiments of DIG fractions showed that the majority of α6β1 integrin was also present in the raft compartment of the newly formed oligodendrocytes, while the αv integrins also expressed on oligodendrocytes [9] were found almost entirely in the nonraft compartment (Figure 1D). We believe this to be the first direct demonstration that the α6β1 integrin is localized in lipid raft microdomains, although recent studies have shown that β1 integrins [10, 11], αLβ2 (LFA-1) [12, 13], and αvβ3 integrins [14] are associated with lipid raft microdomains in other cell types.

Biochemical preparations of lipid rafts obtained by density centrifugation most probably contain a complex mixture of different lipid rafts, each of which may contain different signaling and other proteins [15–17]. To determine whether the integrin and growth factor receptors whose interaction enhances survival signaling were present in the same set of rafts, we used immunofluorescence to examine the distribution of lipid rafts, α6β1, and the PDGFαR in oligodendrocytes grown on Ln-2, Fn, and PDL substrates. To visualize lipid rafts, we used cholera toxin subunit B (CTB), which detects clustered GM1 ganglioside, a marker for a subset of lipid rafts [18]. Double immunofluorescence staining of GalC-stage oligodendrocytes with anti-PDGFαR antisera and FITC-conjugated CTB showed extensive colocalization of both molecules on PDL, Fn, and Ln-2 (Figure 2), consistent with the association of PDGFαR with GM1-containing lipid rafts. Conversely, double immunofluorescence with anti-α6 antibodies and FITC-CTB showed that α6β1 was excluded from GM1-containing lipid rafts on PDL and Fn (Figure 2) and appeared as patches in

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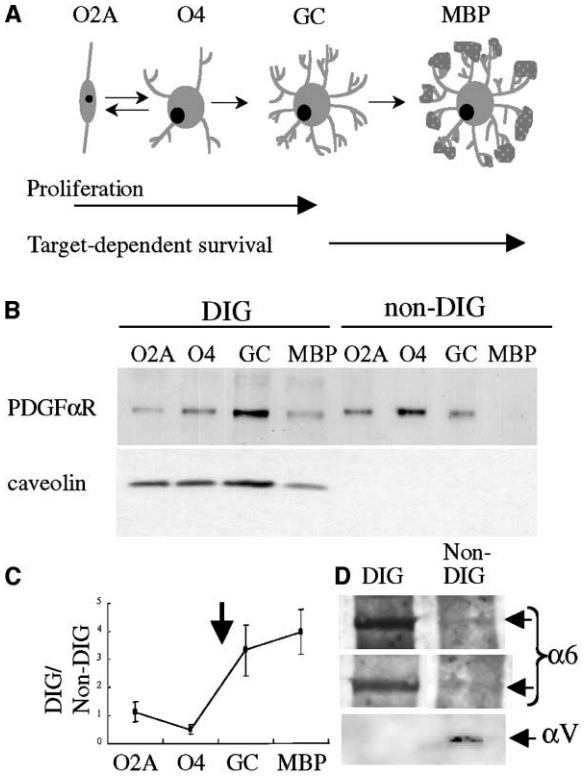

Figure 1. PDGFαR and α6β1 Integrin in Lipid Rafts during Oligodendrocyte Differentiation

(A) Simplified scheme of oligodendrocyte development. The typical morphological changes and expression of cell stage-specific markers representing the four major developmental stages used in this study are shown, with the timing of the transition from cell proliferation to target-dependent cell death.

(B) Oligodendrocyte precursors were differentiated by removing growth factors as described in the Supplementary Experimental Procedures (available with this article online) and harvested at the different developmental stages. DIG and non-DIG fractions were prepared using Optiprep step-density gradient centrifugation. The expression of the PDGFαR was analyzed by immunoblotting followed by ECL detection. Caveolin was used as a positive control for the presence of DIGs, as caveoli copurify with lipid rafts in these preparations. Note that the relative levels of the PDGFαR in the two fractions were equivalent in the precursor cells, but were significantly enriched in the DIG fraction following differentiation.

(C) Results from three experiments are quantified, with the arrow representing the stage of differentiation.

(D) Growth factor-expanded oligodendrocyte progenitors were differentiated to newly formed oligodendrocytes (GalC) and harvested, and DIG and non-DIG fractions were prepared as above. α6β1 and αv integrins were visualized by Western blotting and ECL detection. Two independent experiments are shown for α6β1 and one experiment for αv; note the significant enrichment of α6β1 within the DIG fractions, while αv is enriched in the non-DIG fractions.

the primary processes between CTB-labeled areas. As our biochemical data show that the majority of the integrin is present in a lipid raft compartment at this stage (Figure 1D), we conclude that most of the α6β1 is present in GM1-negative rafts that are not labeled by CTB. In contrast to the results on PDL and Fn, however, on Ln-2 substrates we observed a partial overlap of integrin α6β1 with the GM1-containing lipid rafts (Figure 2). Confirmation of an interaction between α6β1 integrin and the PDGFαR came from immunoprecipitation experiments. When DIG preparations were immunoprecipitated with anti-PDGFαR antisera and then Western blotted with anti-α6β1 integrin antibodies, the integrin was present in association with the growth factor receptor only on Ln-2 substrates (Figure 3A). We conclude, therefore, that Ln-2 induces a redistribution of integrins in the membrane. As a result, the PDGFαR and α6β1 integrin are now present in the same rafts, although we cannot determine whether these integrins redistribute from GM1-negative rafts or derive from the minority of α6β1 integrins outside the raft compartment (Figure 1D).

The sequestration of the PDGFαR into GM1-containing lipid rafts in association with oligodendrocyte differentiation, and the redistribution of α6β1 into the same lipid raft compartment on laminin substrates, suggests that survival signals could be generated by these receptors in the raft microdomains. Previous work has implicated the PI3K signaling pathway in PDGF-mediated oligodendrocyte survival on PDL substrates [19, 20]. To confirm that the enhanced survival response seen on Ln-2 substrates was also mediated by this pathway, we used pharmacological inhibitors in survival assays for newly formed oligodendrocytes grown on PDL, Fn, or Ln-2. As we have described previously [4], Ln-2 substrates significantly increased survival of newly formed oligodendrocytes in response to PDGF (Figure 3B). Survival on Ln-2, and on control PDL substrates, was completely blocked by wortmannin (WM), an inhibitor of PI3K activity (Figure 3B), as was survival on Fn (not shown). A second PI3K inhibitor, LY294002, gave the same result. In agreement with our previous study, PD098059, an inhibitor of MEK and therefore of p42/p44 MAPK, had no significant effect (not shown). We also examined the phosphorylation of serine 473 in Akt, a well-characterized downstream target of PI3K implicated in survival signaling [21]. To avoid unwanted changes in intracellular signals associated with the longer-term cell adhesion required for differentiation, these experiments were performed on attached oligodendroglial precursor cells 1 hr after dissociation from the rat cortical cultures and before significant cell spreading or process outgrowth is observed on any substrate. The phosphorylation response to PDGF was accelerated by the Ln-2 substrates, being maximal at 10 min as compared with 60 min on PDL substrates, and was then sustained for at least 60 min (Figure 3C). Phosphorylation of Ser^{473} on either substrate was inhibited by WM (Figure 3E). Together, these results therefore provide a potential molecular basis for the survival-enhancing effect of Ln-2 in oligodendrocytes by showing increased PI3K activation associated with PDGF receptor stimulation in this cell lineage.

Having identified PI3K and Akt as being involved in PDGF/Ln-2 signaling within oligodendrocyte precursors, we next determined: first, whether these same signaling molecules were present in the oligodendrocyte lipid rafts; and second, whether raft integrity was required for their function. The p85 regulatory subdomain of PI3K and Akt were present in the nonraft fraction throughout oligodendrocyte development, and in addition levels increased in the DIG fraction of newly formed oligodendrocytes (Figure 3D). pFAK, another potential

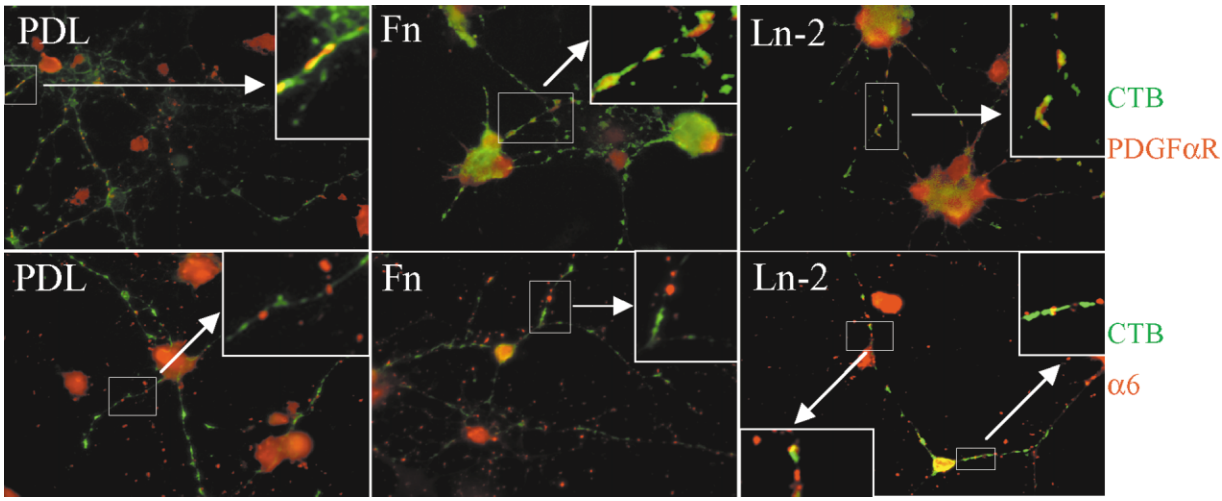

Figure 2. Effect of Laminin-2 on α6β1 Integrin Localization in Lipid Rafts

Growth factor-expanded oligodendrocyte precursors were plated on PDL, Fn, or Ln-2 (10 μg/ml) substrates and allowed to differentiate into newly formed (GalC+) oligodendrocytes. Lipid rafts were then live-labeled at 4°C using fluorescein-conjugated Cholera Toxin subunit B (green), a marker for GM1-containing lipid rafts. The cells were then fixed with paraformaldehyde followed by ice-cold methanol to prevent lateral diffusion of raft molecules within the membrane and processed for indirect immunocytochemistry (red) with either an anti-α6A [24] or an anti-PDGFαR [25] polyclonal antibody. The small boxes at the corners of each panel show higher-power views of the areas indicated by the white box within the main images. Note that α6β1 is distinct from the GM-containing rafts on PDL and Fn substrates, but that colocalization is seen on Ln-2 substrates.

downstream target of both integrin and growth factor receptors, was also present at increased levels in the DIG fraction of newly formed oligodendrocytes (Figure 3D) . To establish whether raft integrity was required for the signaling response generated by PI3K, we examined Ser^{473} phosphorylation after treatment of intact cells grown on Ln-2 or PDL substrates with methyl-β-cyclodextrin (mβCD) to deplete cholesterol and disrupt rafts [22]. As this treatment may only disrupt a subset of raft-associated molecules [6], we first confirmed an effect of mβCD on the raft-associated molecules involved in PDGF/Ln-2 signaling by showing a reduction in PDGFαR, pFAK, p85, and Akt in the DIG fraction and an associated increase in the non-DIG fraction (data not shown). Treatment of either late precursor cells (Figure 3F) or newly formed oligodendrocytes (not shown) with mβCD completely abrogated the enhanced Ser^{473} phosphorylation of Akt on Ln-2, whereas phosphorylation on PDL was less markedly affected (Figure 3F). We have shown previously that process formation in oligodendrocytes is similar on the two substrates [4], so these results cannot reflect a greater degree of cholesterol extraction in cells that have spread more effectively on Ln-2 substrates. We conclude, therefore, that PDGF-mediated oligodendrocyte survival signals are generated in lipid rafts and that the enhanced signaling that results from the integrin/growth factor interaction requires raft integrity.

Our work presented here shows directly that rafts are important signaling platforms for the control of oligodendrocyte survival. More generally, it also adds two new facets to our understanding of raft-mediated signaling. First, it demonstrates a role for rafts in the amplification of signaling by integrin/growth factor interactions. Rafts may provide a mechanism for sequestering together signaling molecules that form specific pathways; for example, the main components responsible for initiation of T cell receptor signaling are present in a subset of T cell rafts [17]. In oligodendrocytes, ECM-stimulated relocalization may allow individual integrin heterodimers or integrin-containing rafts to interact with rafts enriched for molecules involved in PDGFαR-mediated survival signaling such as FAK, p85, and Akt. Second, our work demonstrates a novel role for lipid rafts during development in determining cell signaling responses to growth factors and ECM molecules. Specifically, the redistribution of the PDGFαR, α6β1, and associated signaling molecules provides a mechanism to control independently different aspects of cell behavior (proliferation and then survival) regulated by a single growth factor (PDGF) and both dependent on PI3K activity [19]. The mechanisms that regulate this distribution are unknown, but an attractive hypothesis for the integrins is that activation is responsible. Activation has recently been shown to promote redistribution of LFA-1 and α4β1 integrins into lipid rafts in T cells [13]. We have shown how PDGF signaling promotes proliferation by activation of αvβ3 integrin [23], and a similar activation of α6β1 and subsequent raft relocalization provides a potential mechanism to explain integration of survival signaling.

Supplementary Material

For supplementary data containing the Experimental Procedures, please see http://images.cellpress.com/supmat/supmatin.htm.

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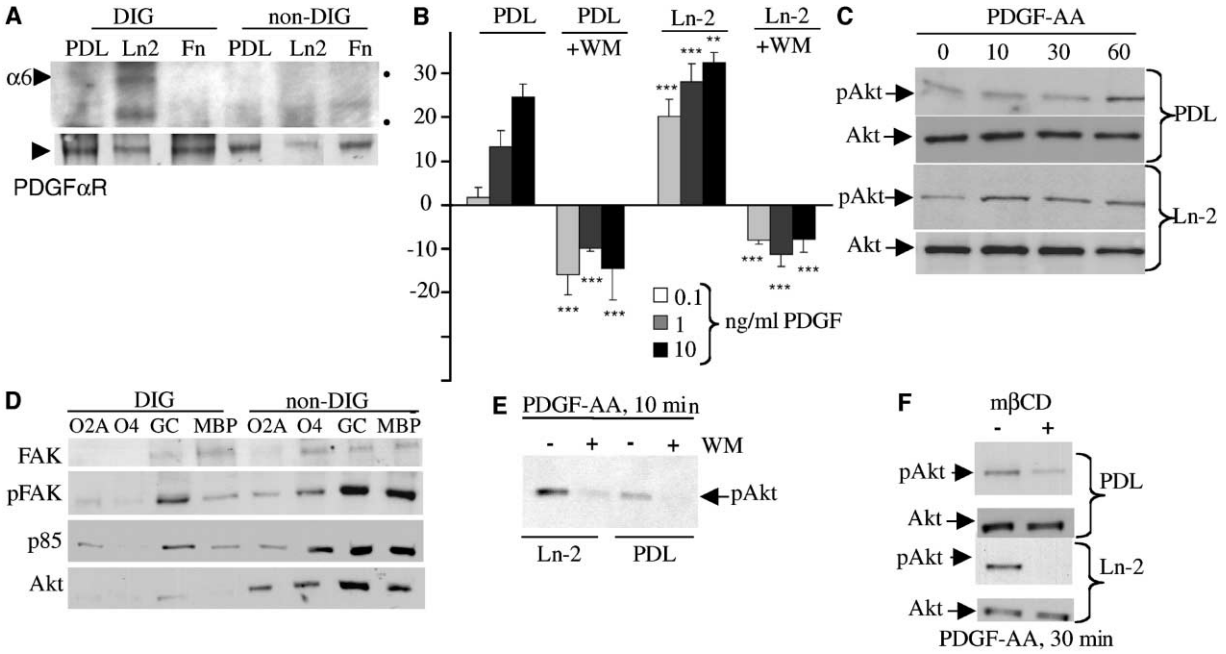

Figure 3. Characterization of PDGF-Mediated Survival Signaling Pathways in Newly Formed Oligodendrocytes

(A) Oligodendrocyte precursor cells were plated on PDL, Ln-2, or FN as in Figure 2 and grown in DMEM/SATO medium to allow differentiation into newly formed oligodendrocytes. DIG and non-DIG preparations were immunoprecipitated with an anti-PDGFαR antisera, and the immunoprecipitate was then Western blotted with anti-α6β1 (top gel) or, as a control to demonstrate the presence of protein in all of the immunoprecipitates, with anti-PDGFαR (bottom gel). The majority of the PDGFαR is in the DIG fraction on all three substrates in these newly formed oligodendrocytes, as discussed in the text. 145 kDa and 74 kDa size markers are shown as dots on the right of the top gel. Note that the α6β1 integrin (which runs at 140 kDa) is only associated with the PDGFαR in the DIGs prepared from cells grown on Ln-2.

(B) Oligodendrocyte precursor cells were plated on PDL or Ln-2 and treated with the PDGF-AA concentrations shown with or without 50 nM wortmannin (Sigma) and grown in DMEM/SATO medium for 4 days to allow differentiation into newly formed oligodendrocytes. Cells were then processed for GalC immunocytochemistry, followed by detection of apoptotic cells with a TUNEL assay. Cell survival is expressed relative to cells grown on PDL in the absence of growth factor. At least 100 GalC+ cells were scored as TUNEL-positive or -negative in each experiment, and values shown are % changes in survival expressed as means ± SD of at least three independent experiments. Note the enhanced survival on Ln-2 substrates and the complete inhibition of survival on both PDL and Ln-2 by wortmannin, a PI3K inhibitor. Statistical significance was analyzed using the Student's t test; two asterisks for $p < 0.01$ and three asterisks for $p < 0.001$.

(C) Oligodendrocyte precursor cells were plated on Ln-2 (10 μg/ml) or PDL (5 μg/ml) for 1 hr and then exposed to 10 ng/ml PDGF-AA for the indicated times in minutes. Following lysis in Triton-X100 and SDS, equal amounts of protein were immunoblotted using specific anti-phosphoSer^{473} Akt antibodies followed by ECL detection. Total Akt levels are shown below as loading controls. Note the accelerated and enhanced phosphorylation on Ln-2 substrates.

(D) DIG and non-DIG fractions of the different developmental stages were obtained as described in Figure 1, and equal amounts of protein were immunoblotted with antibodies against FAK, phosphorylated (Y397) FAK, the regulatory p85 subunit of PI3K, and Akt. Note the enrichment of p85, pFAK, and Akt in the DIG fraction of newly formed oligodendrocytes.

(E) Cells were plated on Ln-2 or PDL for 1 hr as above and then exposed to 10 ng/ml PDGF-AA for 10 min with or without 30 min preexposure to 50 nM wortmannin. Cells were lysed and analyzed using the phosphoSer^{473} Akt antibodies, followed by ECL detection. Note the inhibition of Akt phosphorylation on PDL and Ln-2 substrates by wortmannin.

(F) Activation of phosphoSer^{473} Akt on Ln-2 and PDL substrates 30 min after the addition of PDGF-AA (10 ng/ml) with or without pretreatment with cyclodextrin to disrupt the lipid rafts (mβCD, 5 mM for 30 min). Total Akt levels are shown as loading controls. Note that loss of raft integrity prevents the PDGF-AA/Ln-2-mediated phosphorylation of Akt.

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